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How to get off the MX hook

The US Congress has slapped the Administration in the face by denying funds for MX construction: the problem now, for all concerned, is to find a more manageable missile defence.

The Reagan Administration's defeat in Congress on the MX missile is humiliating but well deserved. Since President Carter's time, it has been understood between the executive and the legislative branches of the United States government that this expensive weapon would not be built until some way had been found of deploying it so as to give both generals and taxpayers some assurance that a substantial proportion of the missiles would survive a first attack from somewhere else. President Carter's favourite solution, shuttling missiles about a dedicated subway system, could have been effective if his Congress had not jibbed at the cost. President Reagan's high-tech solution of the same problem — putting missiles so close together that one attack will neutralize the next — is much more ingenious but even less credible (see *Nature* 2 December, p.389). People skilled at the assessment of the damage done by nuclear weapons say that they understand why "dense pack" should be safe, but acknowledge (as if it were a minor oversight) that they should have given other people more time to understand. What the US Congress has now asked is that if, with the benefit of all the briefings to which it has been privileged in the past few weeks, it cannot understand how dense-packed MX could survive, what reason is there to suppose that a hostile power (*any* hostile power) would be so persuaded of the inviolability of some part of an MX force that it would refrain from a first strike?

The same point bears even more directly on what congressmen's (or women's) constituents think about MX. If a missile-park in Wyoming should fall short of seeming to a potential enemy a viable retaliatory force, why should it seem a good investment of tax dollars to the electors in congressional districts? Congressmen (and women) have learned in the past few years, often against their predispositions, that nothing is as certain to crowd out a community hall, or a town square, than an invitation to protest against MX, or nuclear arms as such or, even, against nuclear things (such as nuclear reactors) in an even broader sense. Latterly, the federal government has been brought face to face with this sombre truth by the knowledge that there is a standing army of a quarter of a million people ready to fill the space around the Washington Monument whenever the call goes out for a protest against something nuclear.

So, in their small ways, congressmen (and women) are already individually paying a price for the MX and what is called its "basing mode". The chances are that even the federal government, a loose synonym for the Reagan Administration, will not be able to survive indefinite rebellion against an unconvincing case for nuclear reinforcement. The danger now is that if the Administration insists that its scheme for the deployment of MX is, for all its faults, the best that it can think of, it will merely widen the gulf between itself and those among its taxpayers who dissent. The Administration has to learn to live with its nuclear opposition.

The military have an important part to play in helping to square this essentially political circle. Many of those who disbelieve in Dense Pack do so because they know in their bones that the technical attributes of the MX missile are an impediment to more sophisticated means of deployment. The ideal, of course, would be that missiles such as MX should be made stochastically invulnerable to destruction from some other missile force by their mobility, and by the opportunities for deception that mobility

provides. Putting missiles such as MX in ocean-going submarines may serve well enough for a counter-city force, but the accuracy is not great enough for a missile force intended to knock out other missiles. The simple resolution of this military dilemma, of course, is to acknowledge that MX (which will weigh at least 100 tonnes) is too big to be mobile, and that it would therefore be better to start from somewhere else — say an upgraded version of the submarine-launched Trident, the land-based version of which is known as D-4. Better still, why not go for a much smaller missile that need not weigh more than 10 tonnes or so — mobile enough to be carried on almost any kind of vehicle?

Politically, the US Administration cannot openly embrace such a solution. To embark all over again on the construction of another strategic weapons system would seem like vacillation to the voters and like weakness to potential enemies. Technically, however, there are viable solutions. It would be possible, for example, to build some extra submarines equipped with missiles so as temporarily to keep potential aggressors in their places. That arguments of this kind may have persuaded the Soviet Union, in the mid-1960s, to plan enormously to increase its deployment of strategic missiles is probably, in retrospect, irrelevant. What matters now is merely that the arms race has a curiously irreversible character. If one side builds a new strategic weapons system, the other is likely to follow suit and the result is likely to be a permanent increase of mutual striking power. Building a system like MX is hard enough, but dismantling it is even harder.

Hitherto, the fruits of negotiations on the limitation of strategic arms, such as those that have been taking place at Geneva for almost a year, have been negligible in comparison with what the arms manufacturers have accomplished. Technically, however, there is a way out even from that conundrum: settle for the ratifications of a few simple arms control agreements already negotiated, the threshold test-ban treaty, the agreement to prohibit except under tight controls peaceful nuclear explosions and even Salt II.

The moral, in short, is that if Congress will not give the Administration what it wants, a deterrent that is the opposite of credible in the sense that those who have paid for it do not believe in it, and if (which is not necessarily the case) it is impossible to start again, the Reagan Administration will have to acknowledge that there is no serious alternative to serious negotiations on arms control. As it happens, the Administration has been presented, in the Geneva negotiations on the limitation of intermediate-range weapons in Europe, with an awkward problem on which to brood over the Christmas vacation; the Soviet Union has offered to reduce the number of nuclear warheads based on SS-20 missiles to 300 if only the United States will refrain from putting Pershing II and cruise missiles on their allotted bases in Europe by the end of 1983, and also agree (which is impossible) that the United Kingdom and France should fall in with whatever the superpowers eventually decide. The terms offered are unacceptable not simply because they are unattainable but because they would also be inequitable; they are nevertheless the best prospect at Geneva for a year. The United States should recognize the offer as an opportunity to throw the theatre and strategic weapons negotiations together (where they belong), for winning some arms control agreement below present levels and for escaping from the MX hook.



Protecting basic research

The US administration plans a generous science budget. It must not look for quick returns.

Curious things are happening at the National Science Foundation, where the ebullient Dr Edward Knapp took over as director a few weeks ago (see *Nature* 11 November, p.100). The good news, or at least the advance reports thereof, is that the federal government's budget for the year beginning next October, to be published when the new Congress has convened in January, will ask the Congress to approve a substantial increase in the foundation's budget by about 18 per cent, well above the rate of inflation (now down to 6 per cent). This development seems to be but a part of the Administration's determination that whatever happens in the next year or two ahead, basic research will not be starved of funds. The principle seems to be that while systematically ridding itself of obligations to carry through further technological demonstration projects — Clinch River is an exception — on the grounds that industry itself should pay for potentially money-making projects, the Administration appears to have acknowledged its own continuing responsibility towards basic research. Other agencies than the foundation, the National Institutes of Health in particular, will now be anxious to find out if the Administration's new-found generosity applies to them.

The other side of the same coin is that the Administration is looking for results — prosperity and all that — but results that it cannot yet clearly define. The argument seems to be that if the Administration has been so good as to agree with academic scientists that basic research is ultimately the wellspring of industrial innovation, and has written its cheques accordingly, it is perfectly within its rights to ask that innovations should flow thick and fast once the cheques are in the mail — and preferably before the next election twenty months or so from now. In the circumstances, it is understandable that Dr Knapp should have been advised to get rid of the three assistant directors whom he inherited (see *Nature* 16 December, p.567); the well known phenomenon that new brooms are rendered ineffectual by the tired servants who use them (to mix a mixed-up metaphor) is in this case complicated by the Administration's need to know who will be responsible for what befalls in the years ahead. The changing of the guard at the foundation is, as of now, politic and not political; Dr Knapp, supported strongly by his previous colleague at Los Alamos, Dr George Keyworth (now the President's adviser on science and technology at the White House), is promising to change the world, perhaps even to break the mould or shift the paradigm; those who will be writing the cheques in the fiscal years ahead want to know who should be blamed if Dr Knapp fails to deliver.

Knapp's prospectus is startling not merely because of the promises it makes on behalf of basic science as the fountain from which innovations spring but because it acknowledges that basic research is educative. Only two years after having offended almost everybody in sight by deleting from the foundation's budget those line-items concerned with the support of science education (in translation, the equivalent of curriculum development in the 1960s mould), the Administration is about to go to Congress saying that the National Science Foundation has a part to play in the education of scientists but this time at a professional level, perhaps by means of partnerships between industry and universities that will be sweetened by modest support from the National Science Foundation. Some of the schemes now being canvassed in Washington are not very different from those tried out in the past decade by the Science and Engineering Council in the United Kingdom. If that is how the budget indeed turns out, the consequence will be that even the next administration (due to be elected in 1984) will not know what to make of Dr Knapp's promises.

What follows is what is called normative or prescriptive advice. Understandably but also rightly, Knapp is impatient that so little (in the way of innovation) has been accomplished by so much expenditure. Among such people, impatience is a virtue. Academic scientists, the foundation's chief pensioners, are

almost wilfully indifferent to the needs of industry, their students obstinately persuaded that the academic life is best. A direct attack on these familiar conventions from somebody such as Knapp could help invigorate the system by means of which institutions of higher education train professional scientists. The result, with luck, could be not so much a spate of industrially relevant innovation as a modest cadre of able people. But none of these benefits would show up within what politicians would consider a reasonable time, and certainly not before the next election. More might be accomplished through the National Institutes of Health, which have at least a chance of understanding (not curing) cancer. So Dr Knapp must now keep talking (in public) about other people's opportunities in the hope that he will eventually be recognized as the man who made the National Science Foundation into what it should always have been — a low-budget agency with disproportionately large responsibilities.

Inventing the Wheel

The impending inquiry on nuclear power in Britain promises frustration.

Some years ago (to be precise, in 1972) the Central Electricity Generating Board, the only British public utility empowered to generate electricity for sale to commercial buyers, told a parliamentary committee of its hopes for the future of nuclear power generation. With electricity consumption increasing by seven per cent a year, or doubling every decade, and with the stagnation of coal output, it seemed natural enough that the board should rely for its future sources of energy on nuclear power and oil. (The last strand in the British fuel economy was cut in 1973, when the price of oil was multiplied by between three and four.) In 1972 the generating board was able, a little controversially, to announce that among the nuclear plants it planned to build would ideally be one fashioned on the principles laid down by engineers in the United States, in which the uranium fuel would be enriched to the extent of a few per cent in uranium-235 and in which heat would be carried away to steam generators by means of water under considerable pressure, perhaps 100 times that of the atmosphere. Next year, on 11 January 1983, a public inquiry will be opened in the country town of Sizewell in Suffolk so as to enable the British government to decide whether what is essentially a carbon copy of several hundreds of reactors built elsewhere should be built in Queen Elizabeth's fair isle. With a little luck, construction might begin fifteen years after the first intention was announced.

The inquiry is a public foolishness for which nobody can be excused. The chief culprit, however, is Mr Tony Benn, Minister of Energy in a previous British government who decreed that nothing should be done at Sizewell without a full inquiry. (The present British government weakly assumed Mr Benn's obligation at its election in 1979.) The Central Electricity Generating Board, which has steadfastly refused to confirm the obvious — that the inquiry is a waste of public money and a needless impost on electricity consumers — is almost as much to blame. For a single pressurized-water reactor is not in itself a hazard — and the best way of telling what the hazard would be if there were several such reactors is to build one of them in the first place.

None of this implies that the British government and the public monopolies that it franchises are neglectful of their duty; rather, the opposite is the truth. During the past three years, all those concerned have leaned over backwards to accommodate objectors to the proposed reactor, with the result that quaintly named organizations such as Friends of the Earth have taken to complaining that nobody will pay their expenses to travel in January from their headquarters (usually in London) to Suffolk (less than 100 miles away) to repeat the arguments for which they are already well-known. In much the same spirit might those used to the yoke or litter as a means of transporting heavy objects over uneven ground have petitioned the Roman Senate 2,000 years ago for funds with which to protest at the introduction of wheeled traffic on the newly paved roads.

Missile spin-off creates activity

MX research an ill-wind brings good

Washington

Research centres around the United States will benefit from President Reagan's determination to press ahead with the MX missile. As long as the fate of the 70-foot long missile and its method of deployment hangs in the balance, lots of money must be spent to keep the teams together and to work on the problems under debate.

At the time of going to press, Congress had effectively deleted \$988 million for production of the first five MX missiles, and seemed likely to attach conditions to parts of the \$2,500 million MX research and development budget. But most of this amount seemed likely to go forward, as would other parts of the defence budget that related to research, such as funds for Ballistic Missile Defense (BMD) research.

The Ballistic Missile Office of the Air Force pays out this money, most of it to prime contractors, to engineer and test the components of the missile. As with other major weapons systems, the MX's contractors are scattered around the country (often in the districts of important members of Congress).

The Ballistic Missile Office also buys research from other parts of the Air Force to work on the basic problem of what would happen in a Soviet attack and how many MXs would survive. The computers

that explore these scenarios belong to the Strategic Air Command, but other offices, such as the Air Force's Office of Studies and Analysis (which judges the "military utility" of total weapons systems), do the analysis.

The "super-hardening" of silos is studied by the Defense Nuclear Agency (DNA), a separate Department of Defense agency whose job is to assess the effects of nuclear explosions. In 1982 DNA reported on a series of tests it had made of blasts over super-hardened silos, which showed they could survive. But the tests were made by combining ordinary explosives to simulate the blast of a 25 megaton nuclear explosion, which makes the conclusion suspect, according to Kosta Tsipis, director of the Massachusetts Institute of Technology programme in Science and Technology for International Security.

Two of the national laboratories, Lawrence Livermore and Los Alamos, are expert in the disturbed environment into which nuclear warheads must fly — and so study problems related to the MX basing mode too.

The Army's BMD programme is also gaining research funds as a result of the need to design a system that could defend the "dense pack" missile field. The BMD programme contracts with private universities such as Stanford, Carnegie-Mellon and the Georgia Institute of Technology.

Outside experts who oppose the plan, such as IBM's Richard Garwin, are few, but powerful. If the dense pack plan is rejected, as other, previous basing modes have been, it may be because the research that underpins it is so scattered.

Deborah Shapley

US Congress cops out

Washington

The lame-duck Congress went out with a whimper, not a bang, this week, having failed to act on any of the many science- and technology-related initiatives brought before it this year.

The last hectic hours of the session were taken up with marathon all-night and weekend efforts to agree on a stop-gap bill to keep the government running until September 1983, the end of the fiscal year. The bill that finally emerged from the full Congress, after repeated delaying tactics by Senate opponents of one or another of its provisions, does the following:

● **Clinch River breeder reactor.** \$181

million has been provided to keep this controversial project alive. But opponents, who last week thought they had finally delivered Clinch River a death blow when the House of Representatives voted its funds, did win a freeze on construction and a study of the project's usefulness.

● **Regulation of doctors.** The House-Senate compromise knocks out a rider by the American Medical Association that would have barred the federal government from investigating and regulating the trade practices of doctors and other professionals. The amendment had passed the House, but not the Senate.

The science- and technology-related bills left to die include:

● **Nuclear waste disposal.** The United States still has no comprehensive plan for the construction of a permanent repository from commercial nuclear waste or for the interim storage of spent fuel. A legislative initiative in the last Congress, two years ago, was also killed off at the last minute.

● **Immigration reform.** This legislation would have imposed new restrictions on the employment of foreign students after their graduation from US universities. The bill was defeated in the House largely due to opposition from Hispanic groups concerned about its other provisions.

● **Animal rights.** This bill would have required laboratories receiving federal research funds to upgrade their animal facilities and institutional controls on the use of animals in research. The National Institutes of Health may however adopt some of the bill's provisions anyway.

● **Clean Air Act.** A two-year effort to reauthorize the basic air pollution control law and to add new provisions for the abatement of acid rain failed to get out of committee. The present statute remains in effect.

Stephen Budiansky

Dense pack logistics

On 22 November, the President submitted a "dense pack" basing proposal for the new MX missile to Congress to persuade it to release \$715 million for research on basing modes in the budget for fiscal year 1983 then being finalized. The plan would put 200 of the highly accurate missiles into a 20-square mile area, at an Air Force base in Wyoming, in silos that would be "super-hardened" so as to withstand a massive pressure of 100,000 pounds per square inch. The MX missiles would be packed 1,800 feet apart, so that incoming Soviet warheads would have to burst near each other and destroy one another through a phenomenon known as fratricide. The Air Force, which runs the MX programme, argued that at least 100 MXs in their silos could survive an all-out Soviet attack; because they would be left to retaliate, the Soviet Union would not attack in the first place.

But many in Congress, including conservatives usually supportive of President Reagan's defence programme, attacked

the plan. Bolstered by advice from outside experts, they doubted whether the Air Force could so suddenly have discovered "superhardening", or that fratricide would really occur, or that dense pack would survive exotic methods of attack, such as having the warheads dig up the ground. Moreover, the Air Force admitted that the survivability of dense pack would ultimately depend on building a ballistic missile defence system, something the United States and the Soviet Union virtually renounced by treaty in 1972.

The House voted by 245 to 176 to delete production funds for the missile: the Senate approved the production funds but with a condition that they not be spent until the dense pack plan and the alternatives had been restudied and submitted to the next Congress. The net effect was correction Congress' action was the first serious rebuff to the President's ambitious defence programme.

Deborah Shapley

Canada's seals

Who's cooking what books?

The toughly worded resolution approved by the EEC's environment ministers last week to introduce a ban on the import of seal pup skins by its member states comes amid growing concern that the government of Canada (the country most affected by the ban) is making improper use of scientific data to support its opposition.

The relevant scientific questions concern trends and sizes of stocks of harp seals and hooded seals. A recent statement by the Canadian Fisheries and Oceans Minister Pierre de Bané that an October report by an *ad hoc* working group of the International Council for the Exploration of the Sea (ICES) took a "neutral position regarding

According to one scientist whose work formed an integral part of the ICES review, this conclusion "simply isn't there".

The ICES report estimated stocks of harp seals in the late 1960s at 1.2–1.6 million, while in the late 1970s the estimate is 1.5–2.0 million; it says, however, that the possibility that the herd had in fact decreased over this period was "not negligible". It is now thought that the suggestion of an increase is due entirely to a statistical artefact resulting from an attempt to compare estimates based on different methods.

On the question of the much less numerous hooded seal, the advertisements say "there is no indication that this species is in any way endangered". This contrasts sharply with an earlier study commissioned by the EEC from the British Nature Conservancy Council (NCC), which concluded that there was a risk that both species would be endangered by a continuation of the present rates of exploitation, with the hooded seal being most at peril. The ICES report in fact concluded simply that insufficient data are available to provide estimates of hooded seal stocks. Dr Sidney Holt, vice-chairman of the International Union for the Conservation of Nature and Natural Resources, himself a marine mammal specialist, has described the Canadian claims as "gross distortions" of the study's conclusions.

Mr Brian Casey, First Secretary at the Canadian High Commission in London, said of the NCC study that the conclusions were swayed by bias and that anyway it was invalidated by the ICES study. A Canadian government document also describes the NCC study group as "completely dominated by dedicated opponents of the seal hunt" and says NCC is "flagrantly selective" in its use of data. But the two scientists who drafted the NCC report also sat on the ICES working party.

The questions considered by the NCC and ICES reports were substantially different, and there are in fact few actual points of disagreement. The NCC report considered long-term changes in the total stocks of both species, whereas the ICES report was confined to short-term trends in stocks in the north-west Atlantic. The ICES report actually supports NCC's finding that there is a severe shortage of data on hooded seal stocks, which makes reliable estimates of population sizes impossible. It now appears that it ought also to agree with the NCC's conclusion that it is uncertain whether harp seal stocks are increasing or declining. But all this uncertainty may soon be cleared up. The Canadian government is advocating an International Convention on North Atlantic Sealing to provide for expert study and management of all aspects of the seal hunt.

Tim Beardsley & Jasper Becker

Migrant academics

Mobile Harvard

Washington

Last week, Harvard physicist Sheldon ("Shelly") Glashow, who shared the 1979 Nobel prize, denied widespread rumours that he had been offered a job at Texas A & M university at College Station at an annual salary of \$250,000. But Glashow did say he was considering moving there, had visited the university and looked at property. His wife has also visited. Clinton A. Phillips, dean of the faculty at Texas A & M, confirmed that "we are in a negotiation phase" with Glashow, who may spend a year "to see how he likes it".

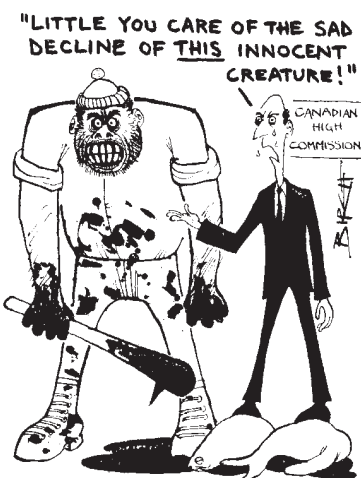
Glashow would be a catch for the university, which sits on the prairie more than 100 miles from Austin and 90 miles from Houston. Twenty years ago, it had 10,000 students and not a strong reputation. But the state had set aside land in West Texas to help provide for the school, and, like other land in Texas, it had oil under it. So in the 1970s, while the endowments of other, better-known universities shrank, this permanent fund that serves Texas A & M and also the University of Texas at Austin, climbed to \$1,700 million — comparable with Harvard's endowment.

Texas A & M has recently lured a new dean to the college of science, John Fackler, a chemist from Case Western Reserve, and provided him with funds for laboratory facilities and staff to continue his research there. Arthur Hansen, formerly president of Purdue University, started as chancellor of Texas A & M last July and was provided with a new home which, Phillips said, cost almost \$1 million.

Phillips believes history as well as money is on his side. "In the beginning in this country there was the Ivy League. Then other universities came to be stars: the University of Chicago, Berkeley and Stanford grew in stature. Now our time has come".

Two things have clouded what otherwise seems to be a case of honest academic piracy. The first was that the university's interest in Glashow became known after it hired a new football coach for a comparable figure including television rights and other benefits. The president and chancellor received rises soon after, just to keep their dignity, so to speak. Nonetheless, the new coach had a bad season.

Texas A & M's arch-rival in football is the University of Texas at Austin, which is entitled to two-thirds of the benefits of the permanent fund. In talking to Glashow, Texas A & M may be trying to rival Austin in physics, too. In January 1982, Steven Weinberg, also a well-known Harvard physicist with a Nobel prize, decided to move to the Austin physics department, re-joining his wife, a professor of law, who in 1980 had joined the faculty of the Law School of the University of Texas at Austin. Weinberg's salary is rumoured to



the hooded seal herd" is described in an EEC memorandum as "utterly wrong and totally without foundation", and catch figures quoted by Mr de Bané are described as "wrong". As to the harp seal, the ICES conclusion — cited in a Canadian government advertisement — that stocks in the north-west Atlantic probably increased between the late 1960s and the late 1970s has since been put down to statistical artefact by several experts.

An attempt three weeks ago by the EEC Environment Council to reach a decision on the proposed ban foundered amid confusion over what legal instrument should be used and what paragraph in the Treaty of Rome should form a basis for the decision. Enthusiasm for the ban ranged from lukewarm (United Kingdom) to fervent (the Netherlands and Italy). The resolution finally approved, unlike a regulation or a directive, is flexible and is not legally enforceable.

Since the abortive meeting of three weeks ago, the Canadian government has placed advertisements in daily newspapers in several European countries claiming that the ICES report, which has been commissioned by Canada and the EEC, "unanimously reported that the harp seal stock of the North West Atlantic has grown significantly during the past decade."

be \$110,000, but he will not confirm this, saying only that his salary is not all that much higher than it was at Harvard.

The university is also giving him an appointment in astronomy and the chance to hire additional faculty to build up the

physics department, which he considers excellent in some areas. "It's a very exciting prospect of being able to build a good group", he says. "I don't believe scientists go anywhere for money."

Deborah Shapley

French nuclear power

Cap de la Hague heads on

The French government has finally approved a major extension of the nuclear fuel reprocessing plant at Cap de la Hague, near Cherbourg on the English Channel coast. Meanwhile, a major and partly critical report on La Hague and on reprocessing in general remains unpublished.

The extension will cost some FF20,000 million (£1,786 million) and covers the upgrading of the existing UP2 400 plant to handle 800 tonnes of spent pressurized water reactor fuel and the construction of a completely new plant (UP3) to handle the same amount. The plants would handle French spent fuel and that arising from plants in Japan, West Germany and Belgium with which the French fuel company Cogema already has contracts. Without the extension, the French nuclear power programme would have been compromised. But UP2 400 has been notoriously ineffective — reprocessing only 250 tonnes in its first five years compared with its projected 2,000. After a series of accidents, one of them potentially disastrous (see *Nature* 290, 538; 1981), the present government set up a scientific commission to investigate the plant.

The report is now complete and should be published in mid-February — but the government has announced that the expansion of La Hague should go ahead

before the report is out.

The commission's conclusions, although not public, are believed to be broadly in line with remarks communicated by Professor Castaing, its chairman, to science minister Jean-Pierre Chevènement in April. These were that while Cogema and the Commissariat à l'Energie Atomique now "have the know-how" to build the extension, the technology is only sufficient "for the short and medium term". Castaing told Chevènement that the most satisfactory solution would be "better waste treatment", in particular the isolation of neptunium and americium from the high-level waste stream. The chairman also argued that long-term storage of unprocessed spent fuel, either wet or dry, was feasible, safe and economical, and must be studied because part of the spent fuel to which France is now committed might remain unprocessed (if there were problems with the new plant for example). The commission also advocated work on final disposal for glassified waste forms and spent fuel itself. And he added that a fast breeder programme "implies substantial progress in reprocessing". These remarks may have stimulated the government to a preemptive announcement of the La Hague extension.

Robert Walgate

Martlesham in business

An important new semiconductor manufacturing process that was originally developed at British Telecom research laboratories is now being exploited commercially by British Telecom's offshoot, Martlesham Enterprises Ltd, together with Thomas Swan and Co. Ltd.

In January this year British Telecom set up in partnership with other investors to exploit a new process developed by Dr M.M. Faktor and Dr R.H. Moss at British Telecom's laboratories at Martlesham Heath. The process uses a new series of compounds developed in association with Professor D.C. Bradley at Queen Mary College, London, in a technique known as metallo-organic chemical vapour deposition (MOCVD). Existing techniques for MOCVD which used highly toxic and explosive trialkyl gallium and indium compounds had proved extremely hazardous, and had

caused several fires and explosions.

The new chemicals will avoid the use of these dangerous compounds and will also increase the range of applications of the method. In particular the process can now be carried out much more effectively with indium phosphide, a material which is likely to find increasing applications in semiconductor devices which emit and transmit infrared light; connected to optical fibres such devices will be important in long-distance optic information links.

Martlesham Enterprises, after a long search, has now awarded manufacturing and selling rights for the new process to Thomas Swan, a specialist chemical manufacturer, which has already secured advance orders for the new chemicals and process equipment from the Massachusetts Institute of Technology.

Tim Beardsley

Soviet science policy

Spread the net

Softly, softly, decentralization and increased local responsibility are emerging as major themes in President Yuri Andropov's economic policy for the Soviet Union. The annual meeting of the Academy of Sciences of the USSR earlier this month suggested that that decentralization should also apply to science. Matching words to good intentions, coverage of the session in the official party newspaper *Pravda* concentrated not on the speech of the academy president, Academician Anatolii P. Aleksandrov, but on that of one of the vice-presidents, Vladimir A. Kotelnikov, which emphasized the role of the "Republic" academies and the local filials of the All-Union academy.

The occasion coincided with the celebration last week of the 60th anniversary of the founding of the Soviet

INSERM's new shape

French medical researchers are to be subjected to a new form of scientific assessment next year — a mixture of stricter and looser rules than those they face at present.

The new arrangements are part of the long-awaited reform of the Institut National de la Santé et de la Recherche Médicale (INSERM), a reform delayed by arguments over whether directors of research groups should have terms limited to 12 years. That question having been settled — the limit will apply, but not immediately — the way was open for official approval of the full reform. That approval has now been granted, and the details of the reform were published last week.

Among the terms are the new forms of scientific assessment, dear to the heart of INSERM's innovative director-general, M. Philippe Lazar. Lazar has sought a system which would allow non-conformist — and perhaps superficially mediocre — groups to make their mark. He believes talent, particularly in the undervalued sciences, must be given its opportunity, but that judgements, when made, must be strict and scientific.

The reform meets these two objectives. Groups will be judged infrequently (probably less than annually) but firmly by specialist scientific commissions — which must visit the laboratory concerned. The result will be the definition of a research programme, against which the group will be assessed next time round, with a guaranteed long-term budget.

The mechanism will give groups more freedom and more time from pointless form-filling and fund-hunting, Lazar believes.

Robert Walgate

Union as a federal state. (The exact anniversary falls on 30 December.) Formally, Soviet culture is defined as "national in form and socialist in content", although what this means for science is not always easy to say, particularly since the implementation of the "Kruschchev theses" of 1958 which emphasized Russian as the preeminent language of Soviet scientific communication and thus blurred the individuality of such distinctive developments as the "Ukrainian school" of cybernetics of the early 1960s. Some local institutes have, however, achieved international renown, notably the Paton Institute of Electric Welding (Ukraine), the Institute of Astrophysics and Atmosphere Physics (Estonia), the Byurakan Astrophysics Observatory (Armenia), and the Tien Shan Cosmic Ray Station (Kazakhstan). Their reputations, however, derive from their status as representatives of Soviet science rather than of the republic academies to which they belong.

In creating these academies, from the Ukrainian Academy founded in 1919 to the Moldavian in 1961, the Soviet state was following the federal structure of the Soviet Union which formally says that all 15 union republics are fully autonomous. (Significantly, though, the largest republic of all, the Russian Socialist Federative Soviet Republic (RSFSR), has no academy of its own, being presumably well served by the All-Union academy.)

The propaganda value of separate republic academies is clear. In his jubilee speech, Academician Kotel'nikov reiterated the usual half-truth that the Estonian Academy of Sciences was established in 1945, in the process ignoring the 1938 decree of the then independent Estonian Republic setting up that academy.

Recently, there has been an increasing emphasis on the duty of scientific institutes to service local industry. In the remoter areas of the far-flung RSFSR, former research stations have been upgraded into fully-fledged filials of the Soviet Academy of Sciences.

This theme of regional devolution has been evident since before the death of Leonid Brezhnev and the accession of Yuri Andropov as General Secretary of the Party. The media run-up to the jubilee had begun to stress the links between the republic academies and local production. In particular, the influential weekly *Literaturnaya Gazeta* ran a survey in which the presidents of the 14 republic academies were asked to describe some of the most interesting research carried out by their academies, the main difficulties they encountered and the participation of the academies in the present high-priority food programme. The answers concentrated overwhelmingly on local needs — from the use of lasers for environmental monitoring in Lithuania to soil surveys for new vineyards in Azerbaijan. **Vera Rich**

British research policy

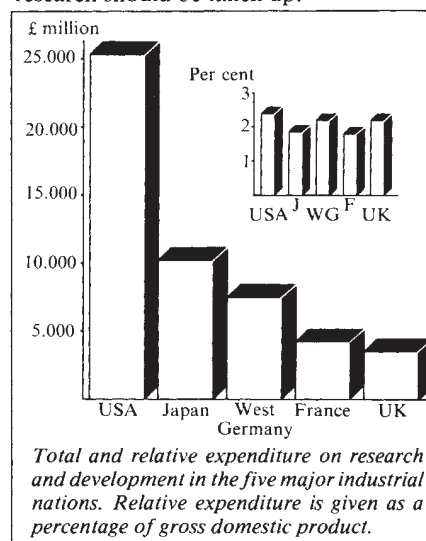
Could do much better

Lack of confidence among would-be innovators was cited last week as a major problem underlying Britain's industrial malaise, by Sir Henry Chilver, chairman of the Advisory Council for Applied Research and Development (ACARD). Sir Henry, who was addressing the Parliamentary and Scientific Committee (an unofficial group of MPs, peers and representatives from industry), stressed the importance of science research and development for Britain, and emphasized the need for effective coordination of policies in the many government departments involved with science and technology.

ACARD's terms of reference were broadened in July and the council is now well placed to play a significant role in coordinating research and development efforts in government and industry. ACARD reports directly to the Prime Minister and will also be advising in the recently announced annual reviews of research expenditure by government departments. Its new role was the result of the government's response to the report on "Science and Government" of the House of Lords Select Committee on Science and Technology, which recommended the appointment of a minister at Cabinet level to speak for science and technology.

Sir Henry pointed out that Britain's expenditure on research and development, while comparable with that of its competitors in terms of percentage of gross domestic product, represented only 7 per cent of the total spent in the five major

industrial nations (see figure). He therefore argued for more spending on research in private industry and a greater concentration of resources in areas with a potential for later exploitation. He also suggested that more ideas from defence research should be taken up.



The primary problem, he said, was one of attitudes, and links between universities and industry should be fostered to encourage academics with good ideas to seek support for commercial development.

Few are likely to disagree, but it is far from certain that university researchers and financial institutions will take notice.

Tim Beardsley

India's second taste of Antarctica

Lucknow

The search for a suitable site for a permanent manned research station is the most important task for India's second scientific expedition to Antarctica. Almost exactly a year after the country's first expedition, the 28-member team set sail on 2 December aboard the hired Norwegian vessel *Polar Circle*.

As with the first mission, the landing site is to be near an old campsite at 70° 3'S/40° 7'E, named Dakshin Gangotri (Dakshin — south: Gangotri — the source of the River Ganges in the Himalayas). An automatic weather recording station has been operating there for the past year and the team's first task will be to collect a cassette of weather data.

The present expedition — under the leadership of Dr V. K. Raina, a glaciology expert from the Geological Survey of India — is due to stay on the ice for 60 days, compared with the 10 days of the first mission. Intelsat telex and telephone links have been set up to maintain contact with India's National Institute of

Oceanography in Goa and the Department of Ocean Development (DOD) in New Delhi. DOD's link with Antarctica is particularly strong, since the head of the first Antarctic expedition, Dr S. Z. Qasim, is now Secretary of DOD.

With plans to have the permanent base operating by 1985, and ambitions either to build or to buy a research vessel of its own, India's interest in Antarctica is obviously growing. India has not yet signed the Antarctica Treaty. Prime Minister Indira Gandhi said in Parliament recently that her government is examining the case for signing the treaty, and she stressed that the implications for all developing countries are being considered.

India is taking part in discussions with other developing countries about joint efforts to explore and exploit Antarctica. However, offers of help from the Soviet Union, which offered the use of Soviet airstrips and buildings on the ice cap, have been turned down.

Zaka Imam

Nuclear reactor safety

Super-Sara's fate undecided

Brussels

Community ministers have again failed to reach a "final" decision on the Super-Sara project at the Joint Research Centre at Ispra in Italy to experiment with loss-of-coolant situations in nuclear reactors. Ever since the project was first proposed after the accident at Three Mile Island in the United States, the Community has procrastinated (*Nature* 11 November, p.99), letting the cost of the project soar.

At last week's meeting, the United Kingdom and West Germany again refused to be placated by Commissioner Etienne Davignon's promises to keep the project within the current estimates of 293.9 million European currency units (ECU) (£161.7 million) up to 1988. Vicomte Davignon, the European Commissioner for Energy and Industry, made a passionate appeal, arguing that it would be irrational to cancel the project when attempts should be made to reassure public opinion over nuclear energy. He recalled that the Western economic summit at Versailles agreed to promote international research into nuclear safety and that there was a danger that, if EEC halted its project, the United States would be unwilling to pass on its own research results.

Davignon held that continuing with Super-Sara would not need extra money apart from an extra 13 million ECU for each year between 1984 and 1987. The extra 27.5 million ECU needed for 1982 and 1983 could be found by transferring resources from another part of the budget. And he thought that by reducing funds for other research at the Joint Research Centres, half of the 39 million ECU needed for the three following years could be found comparatively easily.

The United Kingdom and West Germany were not impressed by this financial juggling and asked whether such a large sum of money might not be better employed in some other way.

The upshot is that a panel of three wise men, independent experts from Denmark, France and West Germany, will review the budgetary estimates and assess the value of the Super-Sara project in comparison with that of similar projects elsewhere. Their report, to be presented at the next research council meeting on 8 February, will also take into account the British delegation's suspicion that Super-Sara will absorb too many of EEC's nuclear scientists in an experiment concerned with coping with an emergency rather than on the prevention of disaster.

In the meantime, the European Commission is to think about alternatives for Ispra if in the end the Super-Sara project is cancelled.

Jasper Becker

Interferon in France

Trial condemned

Paris

Professor Georges Mathé, a well known cancer researcher from the Paul Brousse Hospital at Villejuif, has publicly described as incompetent French doctors involved in preliminary trials with interferon as a possible treatment for cancer. He has criticized the method of administration and the dose and has asked for more information about the circumstances in which four patients involved in the trials died (see *Nature* 11 November, p.97). Mathé has also attacked the establishment where the trials took place and has even asked for the resignation of the members of the scientific council which was set up to monitor the experiments. As a result, the council and IPP (Institut Pasteur Production, the subsidiary of Sanofi-Elf-Aquitaine that produces the interferon) published information about the trials in November. More recently, in a confidential letter addressed to two journalists, they advised wariness of the aggressive tone of Professor Mathé's remarks, which could bring clinical trials of interferon and even

clinical trials in general into disrepute.

Two of the four deaths were from myocardial infarction and another has been tentatively attributed to the same diagnosis, but nobody yet knows for certain what caused them. Professor Flamant, president of the monitoring council, says that it is necessary to keep an open mind about the possible role of interferon. But according to Dr Denis Pouillard, clinical physician at the Curie Foundation who participated in the trials, there was a marked similarity between the symptoms and those he had seen ten years earlier in patients given infusions of leukocytes that had been conserved in poor conditions. Dr Pouillard draws attention to the fact that IPP stores its interferon preparation at 4°C whereas -20°C is generally used for leukocyte interferon storage. He also says that since interferon made up only 1 per cent of the protein in the IPP preparation that was injected, it is likely that proteins other than interferon caused the toxicity.

The accidents gave strength to the supporters of gene splicing methods to produce interferon, which give purer and cheaper products. Professor Flamant, Dr Pouillard and Professor Falcoff at the Institut Curie, who is working with gamma-interferon, all advocate that both methods should continue and their products should be compared simultaneously. They would like to see an international body created to compare clinical trials with different kinds of interferon. At the moment, IPP is performing toxicity trials with the suspect interferon and with a ten-fold purified derivative. If they show that the purified leukocyte interferon is innocuous, clinical trials will begin again, perhaps in January.

Isabelle Trocheris

Toxic wastes blow

Brussels

One of the European Economic Community's hardy perennials has reappeared at the latest meeting of the Environment Council, the battle between the methods used on the mainland of Europe and in Britain for calculating the degree to which industrial discharges of toxic wastes should be restricted. Last Christmas, the British persuaded their partners to include in the directives regulating the discharge of toxic substances into water the "quality objective" method as well as the more strict "limit value" system. The latter sets fixed limits on discharges while the former relates the regulations to the use to which the environment concerned is put.

In the first directive, dealing with mercury, the British conceded that whichever system is used, all new plant must use the modern and effective pollution control equipment.

The second directive deals with cadmium discharges, and the French have been pressing for the British to agree that only limit values should be applied to regulations dealing with new plant. The British claim, however, that this breaks the compromise agreement reached last year.

The issue has now been referred back to Coreper, the committee of the member states' permanent representatives, for further discussion, a considerable setback for the Commission.

Jasper Becker

Jojoba oil

Too late now?

Commercial interest in jojoba oil, once heralded as a substitute for sperm whale oil but since discredited largely because of cost, could be rekindled by a tissue culture technique being developed at Twyford Plant Laboratories in Somerset.

The high price of the oil is largely a reflection of the problems of cultivating jojoba plants. Only female plants produce the oil-yielding nuts and only a small proportion of plants provide a high enough yield of oil to be of commercial value. Attempts to develop the vegetative propagation of high yielding plants have failed because jojoba does not grow well from cuttings.

Now, however, Twyford — part of International Plant Laboratories — and other groups in Israel and the United States have developed a successful approach to propagation by culture of the jojoba's meristem — the growing tip of a shoot or root. The meristem is grown on a series of nutrient media until a seedling develops from it. The seedling is then split into two

or more parts each of which is grown into a complete seedling. By repeating the process a large population of genetically identical plants is produced. And if the starting meristem came from a plant of high oil yield, the population derived from it by meristem culture should also be high yielders.

Twyfod has already shipped clonal populations of jojoba to Arizona for field trials, but admits that the project is still in its infancy, not least because there is a five-year time lag before a plant bears its first crop of nuts.

Even if everything goes according to plan and jojoba oil becomes available at a low price, it is by no means certain that there will be a market for it. According to Highgate and Joab, a major sperm oil refiner now concerned with the development of substitutes, had jojoba oil been available when sperm oil was withdrawn from Britain and the United States, its market would have been assured because it could have replaced sperm whale oil in nearly all of its diverse uses. But industry has since become accustomed to using different oils for different processes and today's market will simply judge whether jojoba oil is better than any of these oils for a particular application. The most likely future for jojoba oil now lies in small specialized fields where no other reasonable alternative exists. **Melanie Kee**

Cancer prize

Washington

The Hammer Prize Foundation has awarded its first \$100,000 prize for cancer research to Dr Ronald Levy of Stanford University and George Stevenson of the University of Southampton (in Britain). They were cited for their work on monoclonal antibodies in the treatment of B-cell leukaemia and lymphoma.

The prize, which will be awarded annually for ten years, was established by Armand Hammer, the chairman of Occidental Petroleum. Hammer has offered an additional \$1 million for a "cure" for cancer similar to the cure for polio achieved by Dr Jonas Salk. The annual prizes are for the scientists who have made the "greatest contribution towards a cure".

The prize has stirred a certain amount of controversy, both for its origins and for its terms. Some researchers question its focus on treatment, rather than basic research, saying that such an emphasis encourages "premature jabs". And there has been grumbling, too, about Hammer's ties with Occidental, the parent company of Hooker Chemical of Love Canal fame. Hammer's appointment to head the President's Cancer Panel — previously a panel of medical experts — had earlier provoked similar criticisms. **Stephen Budiansky**

Chemical toxins

USA and UN disagree

Washington

The issue of the use of Soviet toxic weapons in South-East Asia remains disputed. While the US State Department continues its campaign to convince sceptics that Soviet toxin weapons have been used in Afghanistan and South-East Asia, a long-awaited report by a United Nations (UN) group of experts has provided only inconclusive findings.

The most dramatic pieces of evidence now produced by the State Department are two Soviet gas masks obtained in "clandestine operations" in Afghanistan. According to the US Army Chemical Systems Laboratory, which analysed the masks, traces of several trichothecene mycotoxins were found on the surfaces and hose connections of the masks. These fungal toxins are said by the State Department to have been used extensively in Laos and in Kampuchea, where "yellow rain" attacks were first reported.

The State Department has also released analyses of blood, urine and tissue samples from 33 alleged victims of toxin attacks; trichothecenes were found in 16 of the subjects. "The finding of T-2 toxin and HT-2 toxin (a metabolite of T-2 toxin in animals) in the blood, urine and tissue of victims of these attacks provides unequivocal evidence of their use as weapons", the State Department concludes.

But in its report to the UN Secretary-General, the group paints a far more complex picture. Most striking is the utter confusion that the group found in refugees' reports of attacks and claimed illnesses. Two Hmong refugees who said they developed a rash after a chemical attack two weeks earlier were found on examination to have three-month-old fungal infections. Some others who attributed their illnesses to chemical weapons were found to have malaria, or in one case, a gastric ulcer. The group also found discrepancies in reports of numbers of fatalities, the colour of the agent said to have been sprayed and the timing and seriousness of the effects. The group's work had, however, been complicated by the denial of access to Laos and Kampuchea and was limited to interviewing and examining refugees who had crossed into Thailand long after the alleged attacks took place.

The most the UN group was willing to concede was that there is "circumstantial evidence" for the use of "some toxic material" in the areas of Laos inhabited by the Hmong. The group found stories of chemical weapons use in Afghanistan less believable, attributing reports there to incendiary weapons (which would give off irritating smoke as a by-product of combustion) and to a harassing agent such as adamsite. And reports of rapid decomposition of the bodies of chemical attack

victims — which have been given prominent attention by the State Department — were "difficult to reconcile with known facts about the effects of chemical warfare agents or other chemical compounds".

Chemical analyses performed for the UN study were likewise inconclusive. Two of the laboratories that analysed blood and leaf samples from Thailand were unable to detect any trichothecenes — even in a control sample spiked with the compounds. A third laboratory reported trichothecenes in all samples submitted, including a blank control.

Furthermore, the possibility of cross-contamination of samples is always present; in addition, the identification of trichothecenes is to some degree a function of the analyst's experience and judgement.

The State Department has tried to avoid argument about its analyses by sending samples to Dr Chester Mirocha, a leading authority on the analysis of trichothecenes. Mirocha's technique is to react samples with trifluoroacetic acid; this produces volatile derivatives of trichothecenes that may then be separated by gas chromatography (GC) and analysed by mass spectrometry. A combination of the GC retention time and the mass-spectrometer peaks can provide "certain" identification, Mirocha says, with a detection limit of less than one part in 10^9 . The blood and tissue samples that he analysed contained trichothecenes in the range 10–100 parts in 10^9 .

Mirocha points out that even among workers in laboratories with a great deal of experience, quantitative results of trichothecene analyses can vary by as much as 30–60 per cent; in experienced laboratories the variations would not surprisingly be much greater, he says. "Some people have come into this entirely cold. Just because they're chemists doesn't mean they can automatically analyse for trichothecenes", says Mirocha. The UN group used laboratories at the Tokyo University of Science (run by Dr Y. Ueno, who, along with Mirocha, is considered to be one of the world's experts), the University of Veterinary Medicine in Vienna, and the Technical Research Centre in Espoo, Finland (see *Nature* 16 December, p.569).

But reliable as Mirocha's analyses may be, several independent experts question seriously what exactly he has been analysing. The gas masks, for example, were first analysed at the Army's laboratory; Mirocha was sent only a chloroform extract and asked to confirm the presence of T-2. Mirocha says he was also sent a piece of rubber or plastic on which he was unable to detect any of the toxins.

The State Department refuses to say how the masks were obtained, and independent verification of their origin and handling is

clearly impossible. Gary Crocker, an intelligence analyst for the State Department, did say that they were obtained in "clandestine" operations specifically planned to obtain evidence of the use of chemical and toxin weapons. One mask was removed from a dead Soviet soldier in Afghanistan in December 1981; the other was obtained in Kabul, Afghanistan, in September 1981. Crocker says "the people who got it were instructed on how to handle it", and were provided with a similar sample container that would prevent contamination. "Things were handled properly all down the line".

Dr H. Bruno Schiefer, who conducted a study of toxin weapons use in South-East Asia earlier this year for the Canadian government (a study that found effects that could not be explained by "naturally occurring phenomena"), says "It doesn't really matter who" provides samples — the results "still don't stand up in court". Schiefer says "we would need some sort of verification" by scientists who could go into the affected areas, collect samples and bring them back for analysis.

Stronger criticism comes from Arthur Westing of Hampshire College. "The US has a long history of doctoring evidence", he says. "I don't know if the State Department is lying, but they haven't come close to proving their case." Westing says that "the most elementary mistake they made" was not to get blood and urine samples from a control population outside the area where toxins were allegedly sprayed. The parts-per-billion levels of trichothecenes found in the medical specimens from alleged victims may, he says, reflect natural exposure to the toxins: "There's no question that the *Fusarium* fungi that produce these toxins do occur in that region. The *Fusarium* fungus responsible for T-2, for instance, was described in the 1930s as occurring in Vietnam, according to Westing. And Westing points out that aflatoxin, well known to contaminate food, was found in one of the alleged victims at a concentration comparable with that of T-2.

Mirocha counters that his experiments with cows show that relatively large amounts of T-2 — hundreds of milligrammes — have to be ingested before it shows up in the blood at even the 10–100 parts in 10⁹ level. "To say it's present endemically in the food is absurd", he says.

The State Department, for its part, seems less concerned with technical details than with the overall pattern of evidence. "I could take any piece of evidence and give you an alternative hypothesis", Crocker says, but the "strength of the case" lies in the agreement among reports from refugees and defectors, intelligence information and laboratory analyses. That argument is not likely to convince many sceptics; definitive proof may come only when an independent group of experts is allowed into Laos and Kampuchea.

Stephen Budiansky

Tasmanian environment

Dam threatens wilderness

Canberra

An area rich in archaeological sites from the last ice age will disappear beneath a flood of water as part of a hydro-electricity project unless Australia's national government overcomes its reluctance to interfere with a state decision. Last week Unesco's World Heritage Committee, meeting in Paris, placed the Western Tasmania Wilderness National Park on its register, thus increasing the pressure on the Tasmanian state government to reconsider its plan to build a dam on the Gordon River, just below its confluence with the Franklin River. The dam would inundate 37 kilometres of the Gordon, 37 kilometres of the Franklin and part of all the tributaries of the Gordon including all the recently discovered archaeological sites.

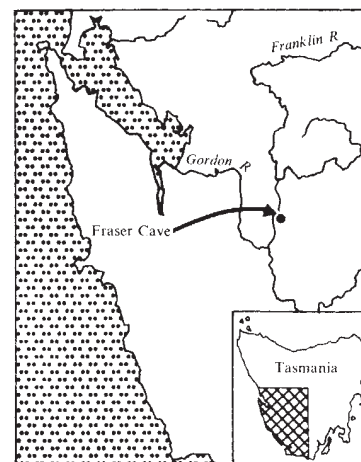
The national government is now left in a curious position. In November 1981 it backed the then Labour state government in applying to put the national park on the heritage list. When a newly elected Tasmanian government later decided to allow the Tasmanian Hydro-Electric Commission to build the dam the national government came under pressure to stop the state scheme. Finally, on 8 December, the federal cabinet decided not to interfere.

Professor John Mulvaney of Australian National University resigned from the council of the Museum of Australia in protest at the decision, arguing that the threatened area qualified for the heritage list under the United Nations convention's criteria for both cultural and natural properties — as well as having archaeological value the area contains an important natural habitat for huon pine trees, some as much as 2,000 years old.

The relationship between national and state governments in Australia is a sensitive issue and the Canberra government is

reluctant to oppose decisions made at state level. In this case the hydro-electric scheme is seen as a way of attracting industry to an area with the highest unemployment rate in Australia — 12 per cent — so it would be a major step for politicians to oppose it.

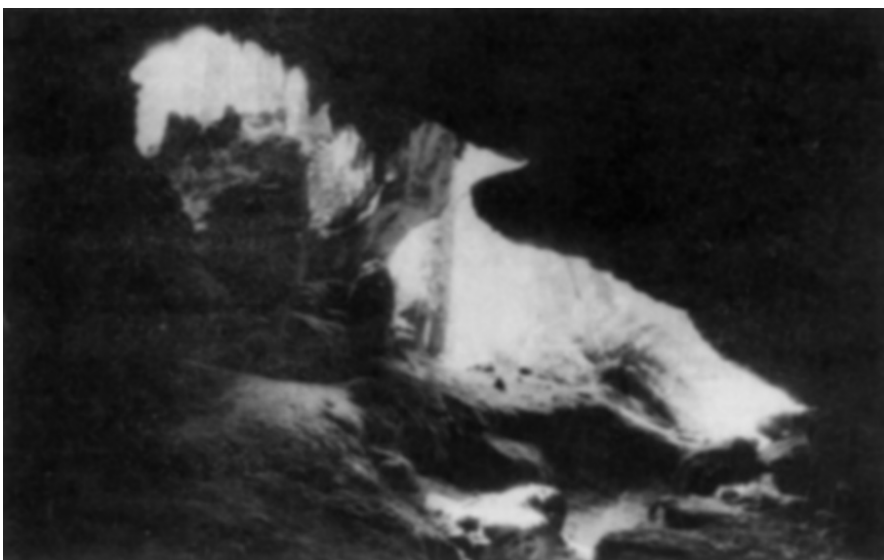
The farthest the government has so far gone in defence of the national park is a statement from Mr Thomas McVeigh, minister for home affairs and conservation, to the House of Representatives,



saying the government "intends to enter discussions with the Tasmanian government with a view to minimizing the damage that the dam might cause . . . it will look towards an agreed management plan for the total area".

The argument that seems to have clinched the decision at last week's Paris meeting is that placing the national park on the heritage list will increase its chances of being preserved. Even so, the Tasmanian government is claiming that the listing gives the dam "the green light to go ahead" — a deliberate misinterpretation it seems.

Vimala Sarma



The entrance to Fraser Cave — due to be submerged if Tasmania's dam scheme goes ahead. In January last year stone tools and human remains dating from the last ice age were found here. The findings are reported in detail in the next issue of *Nature* (6 January 1983, p.28).

CORRESPONDENCE

Pole imprisoned

An open letter to the President of the Polish Academy of Science

Paris, 24 November 1982

SIR — We would like to raise the question of the fate of one of our Polish colleagues, Antoni Stawikowski, a matter which is of great concern to us.

Antoni Stawikowski is a research scientist at the Nicolas Copernicus Centre for Astronomy at Torun. He occupies an important position in the Polish astronomical community, having recently been appointed vice-president of the Polish Astronomy Society. Following his election in 1981 to the position of regional manager of the independent trade union Solidarity he asked for his responsibilities at the research institute to be temporarily suspended so that he could devote his attentions to his union duties.

He was interned on the night of 12/13 December 1981 following the declaration of a state of war in Poland. After a period of detention at Potulice prison he was transferred to the Strzebielinek internment camp. He was freed in August 1982 and returned to his research at the Nicolas Copernicus Centre for Astronomy at Torun.

Now, we have just learned that he appears to have been imprisoned again. We would be very grateful if you would outline the exact motives behind this new imprisonment and specify precisely the nature of the proceedings being brought against our colleague Antoni Stawikowski. We would also like to know his conditions of internment.

Whilst waiting for what we hope will be your rapid reply, we would like you to accept, Mr President, our respectful good wishes.

E. SCHATZMAN
(President)

On behalf of the Council of the Société Française des Spécialistes d'Astronomie, Paris, France

Chinese conundrum

SIR — M.C. McKenna incorrectly implies (*Nature* 18 November, p.212) that, in transcribed Chinese, disyllabic names are all given names and monosyllabic ones surnames. The reverse is true in the name provided for me by Chinese friends: Situ Li. Note also that in *pinyin* romanization there is no hyphen in disyllabic names. Incidentally Chinese names are not the only ones to give problems: a German correspondent once addressed me as "Herr Professor Doktor St. Philip".

PHILIP J. STEWART
Commonwealth Forestry Institute, Oxford, UK

Napoleon's health

SIR — In *Nature* of 14 October there were two contributions that bear relevance upon my own theory of criminal poisoning of Napoleon at St Helena. Lewin *et al.*¹ found no arsenic in a lock of hair obtained by Captain Poppleton, one of the British officers at St Helena. The sample did contain a moderately increased level of antimony, however.

Napoleon's health improved considerably during the first 9 months of his captivity. The

length of hair that grew on Napoleon's head during this period can be estimated at 9–10 cm. The lock of hair obtained by Poppleton may well have been from this period.

It is more difficult to explain why the analyses showed an increased level of antimony. It is true that antimony is a component of tartar emetic, which was used as a medication, but it is also known that Napoleon stubbornly refused to accept medicines. As far as we know, Napoleon had no symptoms of antimony poisoning before March 1820. If the results of the analyses are correct, one is forced to question the authenticity of the sample.

One statement by Lewin *et al.* needs correcting. My theory of arsenic poisoning^{2–4} was not based mainly on the high contents of arsenic found in hairs from Napoleon, but mainly on written evidence (diaries, letters, postmortem reports). The results of analyses of hairs must be seen as a confirmation.

Jones and Ledingham⁵ found arsenic in wallpaper from the drawing room of Longwood. The authors mention that mould on damp wallpaper can metabolize arsenic compounds to volatile arsenic trimethyl. Leaving aside the question of how damp Napoleon's wallpaper was, the fact remains that the clinical picture is not one of continuous low-level poisoning. Napoleon repeatedly suffered from very severe attacks of illness; these were separated by periods of recovery. Analyses of hairs confirm that Napoleon repeatedly consumed relatively large amounts of arsenic.

For about 15 months, from July 1819 to September 1820, Napoleon enjoyed a relatively good health. During this period he could again go for walks, take part in gardening, and go horse riding. Napoleon's disease proceeded independently of any constantly active cause.

Furthermore, Napoleon did not live alone in his house. He was surrounded by an impressive staff of Frenchmen. The diaries do not report any symptoms of chronic arsenic poisoning among these. All this indicates that the wallpaper was not a major source of arsenic in Napoleon's case.

It is a common misunderstanding that Napoleon was killed by arsenic poisoning. The cause of his death was a combination of a liquid refreshment containing bitter almonds and a large dose of calomel given 48 hours before his death. Napoleon soon fell into deep unconsciousness (the effect of mercuric cyanide). The internal surfaces of his stomach became corroded (the effect of corrosive sublimate). The main purpose of the slow arsenic poisoning was to give observers the impression that Napoleon had some chronic disease, thus making his death appear more natural. Further details about the death-blow can be found in ref. 3.

STEN FORSHUFVUD

Göteborg, Sweden

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2. Forshufvud, S. *Who Killed Napoleon?* (Hutchinson, London, 1962).
3. Forshufvud, S. & Weider, B. *An Assassination at St Helena* (Mitchell, Vancouver, 1978).
4. Weider, B. & Hapgood, D. *The Murder of Napoleon* (Robson, London, 1982).
5. Jones, D.E.H. & Ledingham, K.W.D. *Nature* **299**, 626–627 (1982).

Napoleon's arsenic

SIR — I wish to make the following comments on the article by Lewin *et al.* in *Nature* of 14 October (**299**, 627–628; 1982) which reported very much lower levels of arsenic in Napoleon's hair than we found (*Nature* **194**, 725; 1962).

The separation we used was a radiochemical one and the interference referred to by the authors as a possible explanation of our detection of high levels of arsenic is below the 1 per cent level. This is easily demonstrated by using the method.

Gamma-spectrometry as used by Lewin *et al.* is a poor method for arsenic analysis. Though for other investigations we use similar equipment (now standard) to that mentioned by the authors we still use the radiochemical separation for trace analysis. We have applied the technique to the analysis of several thousand of standards and samples since the investigation of Napoleon's hair.

HAMILTON SMITH

Department of Forensic Medicine and Science, University of Glasgow, Glasgow, UK

Reprinting history

SIR — Reprints (also known as extracts, offprints) of articles from scientific journals have been used as a form of scientific information exchange for over two hundred years. Scientists have, since the early nineteenth century, acquired and collected them for their personal professional libraries. One well-known reprint collection is that of Wolfgang Ernst Pauli at the European Organization for Nuclear Research, Geneva (a list of it was published in 1969).

In connection with a study of the history of the reprint, I would appreciate hearing from scientists about the earliest reprints they have seen, what nineteenth century collections they may know of which may have survived, and the importance they attach to such collections.

ELLEN B. WELLS

Smithsonian Institution Libraries, Washington DC, USA

Oncogene activation

SIR — The article by Reddy *et al.* (*Nature* **300**, 149; 1982) describes a relatively minor change in DNA structure which results in the activation of the T24 human bladder cancer oncogene. The change is a point mutation which according to the authors is a "mutation of guanosine into thymidine". Obviously, the intent of the authors was to describe a deoxyguanosine to thymidine transition, or better yet a mutation of guanine into thymine. Their error may seem trivial and perhaps amusing, were it not for the fact that such minor differences can have apparently significant consequences on the properties of genes, as shown by the work described in the paper. It is disconcerting that such a fundamental mistake was published in such an important article.

LASZLO BERES

Hull, Massachusetts, USA

NEWS AND VIEWS

Disputes about the Earth's interior

Sir Harold Jeffreys and Dr R. A. Lyttleton are again at loggerheads. Both are mistaken to pretend that plate tectonics does not exist. Lyttleton is right to give another airing to Ramsey's theory.

The most conspicuous of those who reject the theory of plate tectonics is Sir Harold Jeffreys, now 91, who founded the school of geophysics at the University of Cambridge and who, with successive editions of his monumental book *The Earth* (the sixth was published in 1976), has spread his influence far and wide. Jeffreys is not so much an iconoclast as the opposite; far from being prepared to break the icons of the past, he would keep them bright and shining, as can be gathered from his insistence that the successive periods of mountain-building recognized by classical geologists must be explained by a shortening of the Earth's crust in turn caused by the contraction of the body of the Earth in the past 4,500 million years.

As it happens, Cambridge is also the base (some would say refuge) for another distinguished supporter of that view, Dr R.A. Lyttleton, who may nevertheless be better known for his contributions to celestial dynamics and to the theory of comets. So has Cambridge (where Vine and Matthews first demonstrated that the sea floor really spreads (see *Nature* 199, 947; 1963)) become a place in which ideologically motivated men conspire to undermine the theory of plate tectonics? Not a bit of it. Far from making common cause, Lyttleton and Jeffreys have become persistent critics of each other's work. Their discontents, with plate tectonics but also with each other, are nicely illustrated by their latest contributions to the scientific literature.

First, Jeffreys. This month's issue of the *Geophysical Journal of the Royal Astronomical Society* (71, 555-566; 1982) includes Jeffreys's article "Tidal friction; the core; mountain and continent formation" in which he concedes an important point that Lyttleton has been urging on him for the past five years — that in attempting to relate the observed deceleration of the Earth's rotation to the influence of tidal forces, it is essential to allow for the possibility that the moment of inertia of the Earth may be changing. This important issue has its origins in Newton's theory of the motion of the Moon and in Halley's observation, in 1695, that compared with the predictions of that first serious essay in celestial mechanics, the Moon appeared to be decelerating in its orbit about the Earth. By the mid-nineteenth century, the reality of the deceleration (and that of the Sun and planets) had been firmly established and ascribed to the consequences of tidal friction. There are two effects — the Earth's rotational velocity is decreased, but (because angular momentum is conserved) the Moon retreats from the Earth (by about 3.5 cm a year) into an orbit in which the proper motion is decreased.

As it turned out, the first accurate estimates of the quantities concerned appeared only in the 1930s, when Spencer Jones (then the Astronomer Royal of England) was able to distinguish the secular deceleration of the Moon from the apparently random fluctuations of the Earth's rotational speed — a task then complicated by the use of the Earth's rotation as the standard of time. He estimated the secular acceleration of the Moon as -22.4

4.0 seconds of arc per century per century, well within a standard deviation of the values more recently obtained by laser ranging, from the longitudes on the surface of the Earth at which ancient eclipses were observed and by combining accumulated accurate observations of the planets. Jeffreys (like others) prefers a larger value, agrees with Lyttleton that the data do reveal changes of the moment of inertia of the Earth but considers that these may amount to a few parts in 100 million over the course of a decade. The explanation of recent fluctuations, Jeffreys says, is to be found in climatic change and the consequent redistribution of ice and water over the surface of the Earth. But attempts to pin down the long-term non-tidal contribution to the acceleration of

the Earth's rotation should wait on more accurate observations even though the crustal shortening of at least 200 km needed on Jeffreys's view to account for mountain-building must have gone some way to offset the deceleration caused by tidal forces.

Lyttleton has no such inhibitions. His latest book, *The Earth and its Mountains* (Wiley, 1982), published in November, is a splendid *tour de force* intended to bring together his own interpretation of the secular acceleration of the Moon with a theory of the Earth's interior first put forward thirty years ago by the late W.H. Ramsey. As a contribution to the literature, Lyttleton's book has the virtue of being neither a didactic work nor an exercise in popularization but an argument addressed to people in all disciplines able to stomach some elementary algebra. On the basis of the ancient eclipse data, he concludes that the intrinsic acceleration of the Earth's angular velocity due to its decreasing moment of inertia is between a fifth and a quarter of the deceleration expected from the tidal forces of the Sun and Moon. (Lyttleton acknowledges R.H. Dicke's estimate in 1966 of an intrinsic acceleration only a little less.)

Lyttleton bravely and persuasively puts his faith in Ramsey's theory — the view that the molten core of the Earth differs from the mantle above it not in chemical composition but because of a phase transition from solid insulating rock to material of essentially the same composition in a liquid and electrically conducting phase. The theory originally advanced as a means of accounting for the average densities of the terrestrial planets (the Moon included) has the great advantage of consistency and simplicity. One of its particular virtues was to avoid the implausible assumption, current in the 1940s and 1950s, that the molten core consists solely of an alloy of iron and nickel. Shockingly, the papers presented at the Royal Society on the Earth's core in January this year include not a single reference to Ramsey's work (see *Phil. Trans. R. Soc.* 306, 1-289; 1982) even though most recent attempts to account for the composition of the core involve attempts to "lighten" it with the admixture of elements such as oxygen, sulphur and silicon. The chief objection, that shock-wave and high pressure measurements with materials such as olivine have failed to demonstrate the required phase transition, may be irrelevant. Lyttleton is entirely right to carry a torch for Ramsey's theory, which is in that category of theories too neat not to be true. Needless to say, the steady growth of the Ramsey-Lyttleton core neatly accounts for the observed decrease of the Earth's moment of inertia, the contraction of the Earth's diameter in past aeons and thus for mountain-building.

Where does plate tectonics fit in? Lyttleton does not mention this other way of building mountains. Jeffreys refers with regret to the defection of Sir Arthur Holmes to "Wegener's theory" and says that attempts to account for the subduction of oceanic plates must be likened to "cutting butter with a knife also made of butter". Time will probably show that all the participants in this many-cornered quarrel are correct, or partially so. Lyttleton has scored an important point off Jeffreys, but Jeffreys is probably right to insist that the uncertainties about the motion of the Moon and composition of the Earth's core are still too great for a simple resolution of this long-standing dispute. Both of them should also admit, however, that the case for plate tectonics is too strong to be ignored, and both should admit that Ramsey's theory could yet turn out to be more interesting than they acknowledge. In its original version, the theory supposed that the solid rock of the mantle and the molten material of the core were identical in composition. But every chemist knows that multi-component systems in equilibrium with each other may differ in composition.

Onco Gen

from Peter Newmark

A pair of papers in this issue implicate two human genes, both of which have some pretensions to be called oncogenes, in the causation of specific cancers that are associated with chromosomal rearrangements.

The more advanced study is that of de Klein and his colleagues on page 765. They show that *c-abl*, the human gene homologous to the oncogene of Abelson murine leukaemia virus, is translocated from chromosome 9 to chromosome 22 in the leukocytes of three individuals with chronic myelocytic leukaemia (CML). The implication is that *c-abl* is activated as a result of its translocation and that the activation is associated with the disease.

The translocation between chromosomes 9 and 22 is found in over 90 per cent of cases of CML. One result is the so-called Philadelphia chromosome — chromosome 22 without its long arm. Another is the addition to chromosome 9 of the deleted section of chromosome 22. It has also been generally assumed that there is a reverse transfer of material. De Klein and colleagues prove that point by establishing that *c-abl* has shifted from chromosome 9 to the Philadelphia chromosome of CML leukocytes.

Highly suggestive, though this is, of an association between the *c-abl* translocation and the disease, it is only a beginning. Evidence will be needed that *c-abl* is activated by its shift to chromosome 22 perhaps by coming within the active domain of the immunoglobulin λ light-chain genes on that chromosome; and there is the unproven assumption that activation can lead to transformation. The viral gene certainly seems involved in transformation. It produces a tyrosine-specific kinase in Abelson murine leukaemia virus-induced lymphoid neoplasms of mice (although an unrelated transforming gene from such neoplasms transforms NIH3T3 cells in the standard assay for oncogenes — see *Nature* 300, 659) but there is no evidence yet for a similar product in CML leukocytes.

Note also that *c-sis*, the human homologue of another viral transforming gene — that of the simian sarcoma virus — is shifted the other way in CML: from chromosome 22 to chromosome 9.

An even more tentative link between a human homologue of a viral transforming gene and a tumour with an associated cytogenetic abnormality is reported on page 773. The virus is Harvey murine sarcoma virus; the human gene has already been implicated in human bladder carcinoma (see p.762). McBride *et al.* now assign the normal allele of the gene (*c-bas*) to human chromosome 11 and point out that it is deleted in Wilm's tumour.

Caged ATP set free in muscle

from Peter D. Chantler

THE most noble aim of the biochemist, often discussed when inebriate, seldom when sober, is to relate the *in vitro* to the *in vivo*. This is never more true than in the field of muscle contraction where ordered arrays of aligned fibrils and interdigitating filaments have bolstered confidence that this elusive goal could be just around the corner, achievable by a plethora of physical techniques. And X-ray diffraction has come remarkably close. The recent time-resolved studies on isometrically contracting muscle^{1,2} clearly indicate the power of this technique; evidence for a change in crossbridge orientation has been obtained on a millisecond time scale. Despite such advances, the democratic world of atomic masses will inevitably obfuscate a complete correlation of the *in vitro* with the *in vivo*. Other techniques are needed. One approach, due to Goldman, Hibberd, McCray and Trentham (see this issue of *Nature*, p.701), makes use of a novel molecule — caged ATP.

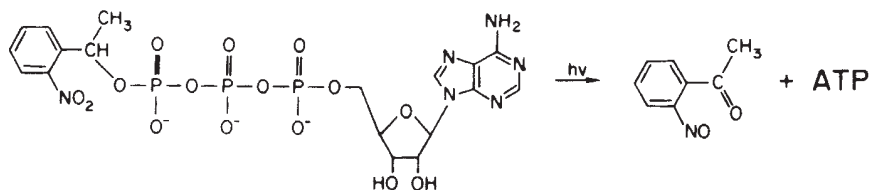
Caged ATP was first introduced by Kaplan and co-workers in 1978 (see ref. 3) who used it to study the ($\text{Na}^+ + \text{K}^+$)-ATPase in resealed erythrocyte ghosts. It is a chemically inert form of ATP, being a nitrophenyl derivative conjugated as the γ -phosphate ester of ATP. Photolysis at the suitable wavelength cleaves the molecule so as to yield ATP and 2-nitrosoacetophenone (see the figure and refs 3,4). The use of a frequency-doubled ruby laser at a wavelength remote from most protein absorption bands (347 nm) demonstrated a linear relationship between the degree of photolysis and the energy output of the laser⁴. Thus it became possible to design an experiment in which a known amount of ATP could be generated in a muscle fibre simply by controlling the total concentration of caged ATP and the energy of the laser pulse. Furthermore, caged ATP does not itself act as either substrate or inhibitor for the ($\text{Na}^+ + \text{K}^+$)-ATPase³ or for actomyosin⁴ — a vital attribute if one wishes to monitor events on a millisecond time scale.

The first experiments performed by Goldman and colleagues (see p.701) were designed to determine the rate of detachment of crossbridges in rabbit skeletal muscle fibres. Single skinned rigor fibres were immersed in a bathing solution (no calcium) containing caged ATP which dif-

fused into the fibre; no change in rigor tension resulted. At zero time, a pulse of 347 nm laser light (30 ns) was applied to liberate a predetermined burst of ATP, resulting in relaxation of the muscle fibre. Both tension and muscle stiffness were monitored. By varying the ATP concentration, the second-order rate constant for detachment could be measured and was found to be $2 \times 10^5 \text{ mol}^{-1} \text{ s}^{-1}$ at physiological ionic strength and neutral pH, identical to that obtained for actomyosin dissociation⁵ (0.5 M KCl) and only a factor of five lower than that for acto-HMM⁶ (0.05 M KCl). The agreement suggests that the detachment rate constant is not substantially altered by the structural constraints of the filament lattice.

The effect of an imposed change in length, one second before the laser flash and consequent ATP release, was also investigated. If the rest-length rigor fibre (no calcium) was stretched 30 μm before ATP release, the initial tension (similar to the force of an isometric contraction) decayed rapidly on photolysis with a half time of less than 10 ms. Alternatively, if the rigor fibre (no calcium) was released by 30 μm before the laser pulse, the lower initial tension either remained constant or even increased before the final relaxation. The final phase of all relaxation transients superimpose. The rise in tension, following ATP generation after a short release step, is not an artefact of the system. Goldman and co-workers present a convincing argument that this effect is a physiological consequence of the type of thin-filament cooperativity first proposed by Bremel and Weber⁷. As noted above, detachment rates determined in the absence of calcium are fast; reattachment rates, determined by performing similar release experiments in the presence of calcium, are also fast and in agreement with X-ray results². If, therefore, the laser-induced production of ATP in the fibre results in detachment of a large fraction of attached crossbridges and yet still leaves a proportion of rigor bonds (myosin heads without bound nucleotide), then even in the absence of calcium, force-generating crossbridges will re-attach because the rigor bonds push tropomyosin

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Cleavage of caged ATP by light.

away from its blocking position⁷. The result will be a transient increase in tension before relaxation. Measurements of stiffness indicate a gradual decline in the total number of attached crossbridges throughout these events, indicating net detachment. As a *piece de résistance* Goldman and collaborators model these release experiments using a three-step cycle involving active, rigor and detached bridges. Model and experiment are in good agreement.

Another approach of particular note, due to Thomas and co-workers, also relates the *in vitro* situation to the *in vivo* one using electron paramagnetic resonance (EPR) spectroscopy⁸⁻¹¹. Spin labels, selectively and rigidly attached to myosin heads in glycerinated rabbit psoas fibres, have allowed the acquisition of data concerning head orientation¹⁰ and submillisecond motion⁹ during rigor and relaxation. The latest paper in this series (Cooke, Crowder and Thomas, this issue of *Nature* p.776) examines the orientation of spin-labelled heads during an isometric contraction. Labelled rigor muscle fibres show a distinctive EPR spectrum indicative of a highly oriented population of heads; a very different spectrum is obtained from the same fibres during relaxation, indicative of isotropic orientation¹⁰. When Cooke and colleagues add calcium to relaxed fibres causing an isometric contraction, the resulting EPR spectrum can be broken down into a summation of spectra from relaxed and rigor muscle fibres in the ratio of 4:1. No other orientations are seen; controls were performed to ensure that all heads were saturated with nucleotide.

Now, the paper of Goldman *et al.* (p.701) puts an upper limit of one per cent on the proportion of time a crossbridge spends as a true rigor bond during an isometric contraction. Hence the observation of 20 per cent spin-labelled heads having a highly ordered rigor-like EPR spectrum during an isometric contraction (Cooke *et al.* p.776) leads one to an ineluctable conclusion: the domain probed by the spin label on the myosin head does not change its orientation during the powerstroke. This conclusion is consistent with the observation that an applied stress imposed on rigor muscle fibres also does not alter the orientation of the domain probed by

the spin label¹¹. Nevertheless, although part of the myosin head may well be immobile during the power stroke, the demonstration of work production by actomyosin machines using only soluble

proteolytic subfragments of myosin (myosin heads)¹² restricts the conformational changes necessary for force generation to other domains on the head itself, rather than the myosin tail. [1]

Transcellular cross-talk between epithelial cell membranes

from Jared M. Diamond

OUR bodies are catacombs of spaces connected to the outside world and filled with fluid. The spaces include the lumina of the intestine, kidney tubules, and salivary and sweat ducts. Separating these spaces from the bloodstream are cell sheets called epithelia, which secrete or absorb fluids by pumping ions from the blood into the spaces or vice versa. An enormous boost to epithelial research came when the Danish physiologists Ussing and Zerahn¹ devised a new method of measuring the pumping of ions in 1951. Seven years later Koefoed-Johnsen and Ussing provided a second leap forward with a new model to explain the pumping². The model has evolved considerably in subsequent years, and this evolution is in turn relevant to the theories of scientific models proposed by historians of science. Several papers published in recent months report further significant advances³⁻⁵.

The Koefoed-Johnsen and Ussing (KJU) model sought to explain sodium absorption, a dominant transport mechanism in many epithelia. In the 1950s, students of nerve, muscle and erythrocytes had discovered how these cells regulate intracellular cation content through three membrane processes: passive permeation of Na⁺, passive permeation of K⁺, and active ATP-driven extrusion of Na⁺ coupled to uptake of K⁺. The KJU model was couched in terms of these same three processes and postulated their restriction to one or other face of the sheet: passive Na⁺ entry to the cell membrane facing the lumen, and passive K⁺ exit and active Na⁺-K⁺ exchange to the cell membrane facing the blood (Fig.1a). Part of the model's appeal was that it postulated no new processes beyond those already known to regulate ion movement between cell interiors and the blood. The limitations of these postulates were also the major focus of the model's further evolution.

Epithelia are not merely polarized cells arranged in a sheet. Each cell in the sheet is joined to its neighbours by junctions, which in principle offer a path across the epithelium that by-passes the cells. The KJU model, and epithelial research of the 1950s generally, ignored the possible importance of this path. In the late 1960s and early 1970s, when new techniques became available, it turned out that the alternative route was not, indeed,

important for one class of epithelia, now termed tight epithelia. In such tissues, exemplified by frog skin, mammalian and amphibian urinary bladders, and stomach, ions cannot pass through the junctions and must traverse the epithelium via the cells. However, in 'leaky' epithelia, exemplified by renal proximal tubule, small intestine and gallbladder, the junctions are the major route for passive transepithelial movement of ions. Tight and leaky epithelia have subsequently been found to differ in the way in which Na⁺ enters the epithelial cells across the lumen-facing (apical) membrane. In tight epithelia, as well as in the medium-tight large intestine and renal distal tubule, Na⁺ enters by a highly specific mechanism that is inhibited by very low concentrations of amiloride and that recent measurements of electrical noise have shown to be an ion channel^{5,9}. In contrast, in leaky epithelia Na⁺ entry is coupled to the entry of a partner (a non-electrolyte such as sugar or amino acid, or Cl⁻), or perhaps to H⁺ exit in parallel with Cl⁻/HCO₃⁻ exchange. This process is believed, but not firmly proved, to involve a carrier^{10,11}.

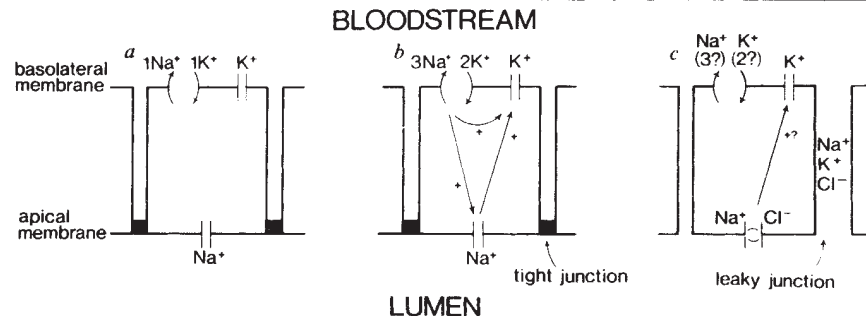
A recent modification of the model came from the discovery that the pump at the blood-facing (basolateral) membrane does not swap 1 Na⁺ for 1 K⁺, as originally assumed, but rather 3 Na⁺ for 2 K⁺, as is also true for nonepithelial cells^{5,12-14}. More major changes have, however, focused on how ion movements at the apical and basolateral membranes regulate each other¹⁵. Apical Na⁺ entry is greatly stimulated by physiological agents such as luminal nutrients, aldosterone and antidiuretic hormone. What prevents the rate of apical Na⁺ entry from exceeding that of Na⁺ extrusion by the basolateral pump, leading to osmotic lysis? Increased basolateral Na⁺ extrusion is coupled to basolateral K⁺ uptake: how is the cell similarly protected against osmotic lysis through increased K⁺ content? Other physiological agents, such as CO₂ and insulin, stimulate basolateral Na⁺ extrusion: what then signals the apical membrane to become more permeable to Na⁺, lest apical Na⁺ entry become rate-limiting? Do conditions that stimulate

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apical Na^+ entry then stimulate basolateral Na^+ extrusion through a rise in cell Na^+ , or is there a different signal? In retrospect, our understanding of epithelial transport in 1958 resembled an economic study of the banking system that considered savings inflow and loan outflow, without having begun to ask how inflow and outflow regulated each other by determining savings and loan interest rates.

The first indication of cross-talk between apical and basolateral membranes came in 1961, when MacRobbie and Ussing¹⁶ used cell swelling as an indirect measure of ion entry in frog skin. They found that inhibiting the basolateral pump reduces the ionic permeability of both the basolateral and apical membranes. The effect on apical Na^+ permeability has now been documented more directly in numerous tight epithelia by electrical methods¹⁷⁻¹⁹. (It has yet to be demonstrated for a leaky epithelium.) Stimulating the pump with CO_2 increases apical Na^+ permeability, while inhibiting the pump by metabolic poisons or by the specific pump inhibitor ouabain reduces apical Na^+ permeability. The effect makes sense teleologically: shutdown of basolateral Na^+ exit leads to shutdown of apical Na^+ entry, protecting the cell against osmotic lysis. But this result, however desirable, is paradoxical. The simplest expectation from shutdown of basolateral Na^+ exit



Evolution of the Koefoed-Johnsen and Ussing model of epithelial transport. *a*, As originally postulated in 1958. *b* and *c*, The present form of the model applied to tight (*b*) and leaky (*c*) epithelia.

would be a rise in cell Na^+ , tending to increase apical Na^+ conductance because of the higher concentration of the conducting ions. In fact, apical Na^+ conductance decreases.

The converse type of cross-talk, a regulation of the basolateral membrane by the apical membrane, has just been demonstrated by two recent papers. In the tight epithelia toad and frog urinary bladders, Davis and Finn³ showed that blocking the apical Na^+ channel with amiloride decreases basolateral K^+ conductance as rapidly as apical Na^+ conductance. In the leaky epithelium *Necturus* small intestine, Gunter-Smith, Grasset and Schultz⁴ found that addition of the sodium ion's transport partners, alanine or glucose, to the luminal bathing solution rapidly increases apical conductance, then more slowly increases basolateral conductance. Furthermore, there is now extensive evidence of feedback within the basolateral membrane: an increase in activity of the basolateral $\text{Na}^+ - \text{K}^+$ pump goes hand-in-hand with an increase in basolateral K^+ conductance^{4,15,19,20}. What remains unclear is whether this basolateral K^+ conductance is part of the pump itself, or whether it arises from a separate channel. It is also uncertain whether a rise in cell Na^+ is the principal means by which increased apical conductance leads to increased basolateral pumping, since searches for the expected rise in cell Na^+ have yielded conflicting results²¹⁻²³.

What signalling agent is involved in these feedback circuits? The leading contender is changes in intracellular Ca^{2+} , normally maintained at a low concentration by countertransport of Na^+ versus Ca^{2+} at the basolateral membrane²⁴⁻²⁶. In other words, Na^+ entry downhill into the cell at the basolateral membrane drives the uphill extrusion of Ca^{2+} , so that reduction of the Na^+ downhill gradient through a rise in cell Na^+ would lead to a rise in cell Ca^{2+} . To explain the observed feedbacks, one would also have to assume that raised cell Ca^{2+} increases basolateral K^+ conductance but decreases apical Na^+ conductance. It remains to be determined whether physiological changes in cell Ca^{2+} caused by changes in cell Na^+ do produce these conductance changes.

All these examples of transcellular cross-talk have involved Na^+ transport. A pos-

sible example for another ion has emerged from studies by Machen and colleagues²⁷⁻²⁹ in gastric mucosa, where increased Cl^- transport is associated with increased transepithelial conductance. The effect appears to involve increases in basolateral K^+ conductance and in apical Cl^- conductance.

Similar interactions between conductances may also appear in isolated cells, as exemplified by an effect of Na^+ entry on K^+ conductance in hepatocytes³⁰. However, it is proving harder to discover feedback links in isolated cells, where the interacting conductances coexist in the same membrane, than in epithelia, where they are conveniently segregated in opposite membranes.

Comparison of Fig. 1*a* with Fig. 1*b* and 1*c* makes clear that the KJU model has evolved considerably since it was proposed in 1958. In tight epithelia (Fig. 1*b*), the pump is now believed to be electrogenic instead of neutral, and three feedback mechanisms have been added. In leaky epithelia (Fig. 1*c*) a major parallel pathway and one feedback mechanism have been recognized, and the apical entry step is now considered not to be of Na^+ alone. These developments illustrate why, in the past two decades, philosophers of science have talked less about the verification or falsification of models, and more about the evolution of models within a continuous framework (for example, the paradigm or disciplinary matrix of Kuhn, the research programme of Lakatos)³¹.

But the history of the KJU model also illustrates that, even at a given stage, a model's acceptance does not depend solely on its verifiability, or on its success in surviving attempts at falsification. Through much of the 1960s and the early 1970s many experimental findings argued against even the basic premises of the KJU model³²⁻³⁸. Why did the model nevertheless remain widely accepted through this period? The fact that today's evidence happens to support the model does not resolve the dilemma, for this outcome was not foreseen from yesterday's evidence.

Several factors may have contributed to this paradox. Although the KJU model conflicted with some important findings, there was never a serious alternative that could begin to resolve these conflicts while matching the successes. Although there

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were doubts about the correctness of the KJU model, its use continued to suggest numerous experiments that yielded significant results. There was concern that the error was not in the model but in the contrary findings: for instance, in estimates of intracellular ion concentrations by indirect chemical means, superseded recently by direct measurements through ion-specific microelectrodes.

Finally, the model was beautiful, simple and reductionist: it made epithelial pheno-

mena emerge from established properties of isolated cells. In contrast, the findings conflicting with the model were complex, intellectually unappealing and unconnected to work on isolated cells. Given a choice between what seemed to be a beautiful model and ugly findings, physiologists preferred the model. Now, many of the ugly findings that conflicted with the model can be explained by our newly-gained understanding of cross-talk. Beauty and truth are once more united! □

Trypanosoma cruzi: signals for transformation

from F.E.G. Cox

Trypanosoma cruzi is the causative organism of Chagas' disease, a disease that affects between 12 and 24 million people in South and Central America and causes considerable mortality and morbidity. The trypanosome is transmitted by numerous species of blood-sucking reduviid bugs. Trypanosomes in the trypomastigote form are taken up by the bug when it feeds and transform into epimastigote forms in the anterior part of the insect's gut. They then multiply by binary fission, building up vast numbers. Transmission becomes possible when the epimastigotes transform back into trypomastigotes again and pass into the hindgut where they are voided with the faeces to enter the victim through abrasions in the skin. The bugs live for long periods and, having become infected, remain infective for life, so providing a continuous threat to all those who might subsequently be bitten.

The signals that trigger these transformations have not yet been definitely identified but it seems certain that the membrane surface of the epimastigote form recognizes signals provided by the intrinsic or extrinsic contents of the insect's gut. The membrane surface is very complex and contains polysaccharides, lipopolysaccharides and glycoproteins. The carbohydrate components can be identified using lectins¹, and peanut butter agglutinin can be used to extract galactose-terminal glycoproteins². Monoclonal antibodies can also be used to identify glycoproteins³. The tools with which to study the signals responsible for transformation in the bug are therefore available.

It has been suggested that the lectins already identified in the insect gut might regulate the morphogenesis of the trypanosome⁴. One of the molecules possibly involved has now been identified⁵ in a letter that has just appeared in *Nature* (Sher, A. & Snary, D. 300, 639; 1982). Epimastigotes do not readily transform

into trypomastigotes (a problem that has also troubled workers using African trypanosomes) and Sher and Snary⁵ devised a technique whereby epimastigotes enclosed within Millipore chambers were transplanted subcutaneously into mice, left in position for 7 h, withdrawn and transferred to an appropriate culture medium. In these conditions, epimastigotes transform into trypomastigotes, a change that does not occur if the *in vivo* 'programming' is not carried out. Using this technique, Sher and Snary were able to investigate the inhibitory effects of six monoclonal antibodies against epimastigote surface components and one lectin, wheat germ agglutinin. The substances to be tested were introduced into the culture media containing 'programmed' epimastigotes and the proportion transformed calculated.

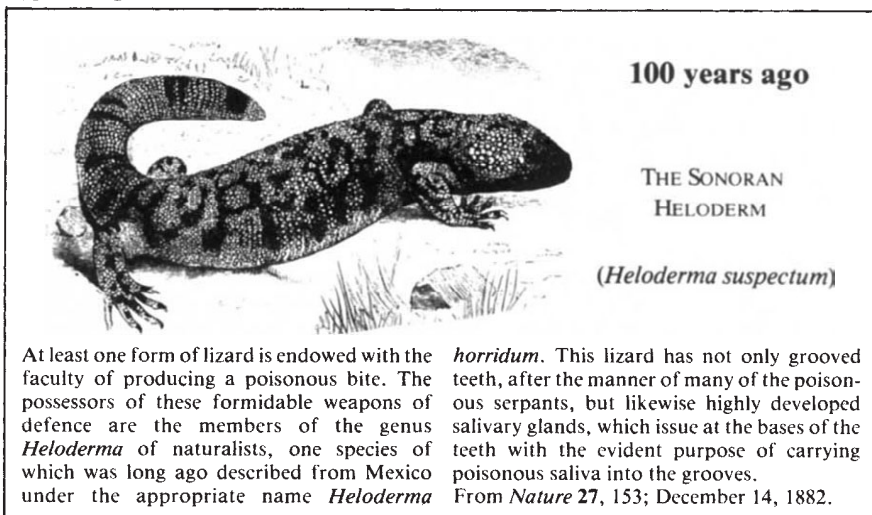
The experiments showed that only one of the monoclonal antibodies inhibited transformation from the epimastigote to the trypomastigote form. The antibody had already been shown to be directed against a cell-surface glycoprotein with a molecular weight of 72,000 (ref.3). It does not affect the longevity or motility of the epimastigote, only its ability to transform into a trypomastigote. The lectin had no inhibi-

tory effect. The results suggest that in the midgut of reduviid bugs there exists a substance, or substances, which reacts with the 72,000-molecular weight glycoprotein on the epimastigote surface and inhibits transformation into the trypomastigote form. Whether or not this is one of the lectins previously described has yet to be determined.

The significance of these findings is that they provide a clue as to why epimastigotes are inhibited from transforming into trypomastigotes. The inhibition probably serves two purposes — to accumulate epimastigotes in the midgut until such time as a relatively large proportion can transform into trypomastigotes, so ensuring the infection of a new host; and to retain a number of untransformed epimastigotes for later transformation and infection. Parasites need to be retained in the midgut because the nutritional conditions are more favourable there than in the hindgut.

From an epidemiological point of view the findings are very interesting. Many bugs can transmit *T. cruzi* but not all do so equally well and it is possible that the potential to act as a reservoir of infection also varies. Susceptibility and the ability to transmit the infection may be tied up with the interactions between surface molecules and insect products and as there are quantitative and qualitative differences between the surface components of different strains of *T. cruzi*⁶ it may well be that a full understanding of Chagas' disease will depend on developments at the molecular level. The findings may also be significant in other situations; in leishmaniasis, for example, lectins have also been shown to bind to the surfaces of the parasites and the capacity to bind to vary between species and strains⁷. Monoclonal antibodies, at present largely the tool of immunologists, may become equally important to epidemiologists. □

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100 years ago

THE SONORAN
HELODERM

(*Heloderma suspectum*)

At least one form of lizard is endowed with the faculty of producing a poisonous bite. The possessors of these formidable weapons of defence are the members of the genus *Heloderma* of naturalists, one species of which was long ago described from Mexico under the appropriate name *Heloderma*

horridum. This lizard has not only grooved teeth, after the manner of many of the poisonous serpents, but likewise highly developed salivary glands, which issue at the bases of the teeth with the evident purpose of carrying poisonous saliva into the grooves. From *Nature* **27**, 153; December 14, 1882.

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Why is the stratosphere dry?

from Reginald E. Newell

MORE than 30 years ago Brewer and Dobson^{1,2} suggested that the stratosphere is dry because air enters from the troposphere only in the vicinity of the cold tropical tropopause. They proposed that the tropical tropopause acts as a cold trap for moisture, limiting the stratospheric moisture to saturation mixing ratio values at the trap temperature. Working backwards from middle-latitude observations the required cold trap temperature is about -84°C . The general theory Brewer and Dobson still holds but the details of the actual troposphere-to-stratosphere transfer are not understood. Does the transfer occur by a steady rising motion or is it part of the active cumulonimbus convection occurring in the tropics? A series of papers³⁻⁹ published in *Geophysical Research Letters* this year gives some insight into the processes involved.

Brewer and Dobson based their theory on frost-point measurements made from aircraft over England, which showed that whatever the conditions in the troposphere, the stratospheric water vapour mixing ratio dropped sharply just above the tropopause to values close to $2\text{ }\mu\text{g}$ of vapour per gramme of air, only one ten-thousandth of the value just above the tropical sea surface. Mastenbrook¹⁰ subsequently adopted Brewer's frost-point hygrometer for balloon use and found similar low mixing ratios in the tropical stratosphere with a seasonal variation generally in phase with the cold trap temperature.

The recent papers in *Geophysical Research Letters* provide the first results from an exploration of the tropical tropopause region by the NASA high-flying U-2 aircraft which was based in Panama during September 1980. The aircraft was equipped with an array of modern instrumentation and made a series of flights to measure temperature, water vapour, aerosols, ice crystals and other atmospheric trace constituents. The results show a water vapour minimum at about 19 km, a few kilometres above the temperature minimum at the tropical tropopause, with mixing ratios down to about $2.2\text{ }\mu\text{g g}^{-1}$, significantly lower than the saturation mixing ratios ($\sim 3.8\text{ }\mu\text{g g}^{-1}$) at the observed tropopause temperatures (around -80°C). The air at 19 km had clearly not entered the stratosphere through the tropopause at Panama. The water vapour was measured with two instruments, a new Lyman- α photodissociation hygrometer⁸ and a more conventional broad-band IR radiometer⁹,

and both showed the same general findings.

Aerosols with radii greater than $0.03\text{ }\mu\text{m}$ were collected by a wire impactor and examined in the laboratory with a scanning electron microscope⁶. It was found that the concentration of small particles diminished and that of larger particles increased with height above the tropopause. The small particles seem to be introduced into the stratosphere by convective activity, though where and when the injection occurs is not known; coagulation seems to be the main mechanism of loss.

The U-2 pilot was bold enough to penetrate the cirrus anvil on several occasions (a somewhat risky procedure as it is hard to see from above where there might be convective updrafts penetrating the lower part of the anvil) and a probe was used to record two-dimensional shadow images of ice crystals in the size range $40\text{--}2,500\text{ }\mu\text{m}$ (ref. 7). In addition, an active cavity laser spectrometer was used to measure the size distribution of aerosols in the $0.1\text{--}0.3\text{ }\mu\text{m}$ size range. Inside the anvil there was a particular abundance of aerosols having a radius of $0.17\text{ }\mu\text{m}$ which Knollenberg and co-workers think is due to new nucleation and growth of solution droplets⁷. This is just one item from the large amount of new data on anvil particles that was collected in Panama.

To accompany the aircraft measurements, enhanced IR photographs of the tropical clouds were produced from the Atlantic Geosynchronous Satellite⁴. These showed the time evolution of the convection and the large extent of the anvils, although there were only a few points where the equivalent temperature was lower than -82°C , in agreement with the aircraft observations.

It had been thought that the moisture in the rising air would condense out to form cirrus cloud and the ice crystals would be left in the troposphere^{1,2}. Danielsen⁵ has examined the new data with this view in mind and interprets it in terms of what he calls 'a dehydration engine'. He describes how a rising cumulonimbus cloud can overshoot the level of zero buoyancy and the anvil form from air that has descended after some mixing with warmer and drier air of the lower stratosphere. The anvil base extends far beyond the parent convective cloud, as shown by the satellite IR images, and is heated by the interception of upwelling IR radiation. The satellite images and aircraft data together suggest an IR emissivity close to unity for the anvil top and this region cools by radiation to space. Static instability is thus maintained in the anvil by radiation and there is a turbulent heat flux from the base to the top for balance. Because the anvil base is about 10°C warmer than the top and saturation

vapour pressure increases with temperature, there is a vertical gradient of moisture and an upward moisture flux is produced by the turbulent transport. Near the top of the anvil there is supersaturation, perhaps even with respect to water according to the observations, and ice crystals grow and fall relative to the air, providing the water sink for the system. The engine mechanism yields a vertical temperature profile with two minima and an intermediate warmer region due to anvil subsidence; the two minima are observed in the flight data. Furthermore, calculations of IR cooling from the ice crystal data and from the IR radiometer data⁷ show cooling rates near the top of the anvil of up to 15°C per day, giving further support to Danielsen's engine concept.

The finding that the water vapour mixing ratio is at a minimum near 19 km, well above the tropopause, and has values smaller than saturation values at the tropical tropopause suggests that air enters the stratosphere in a region where the tropopause is colder than that at Panama. Considerations of the world-wide distribution of temperature indicate that the tropical western Pacific is a region where the exchange might occur, particularly in the December–February season^{5,8,11}.

That possibility, and Danielsen's concept of a 'dehydration engine', could be tested by carrying out a similar experiment in that region, and with more extensive radiative measurements it should be possible to check Danielsen's predictions of a radiatively produced instability. Even in the western Pacific convection can only dehydrate the air just above the tropical tropopause. The dry air seen at 19 km at Panama must have risen by a gentle rising motion that would accompany radiative heating of the region above the convection and this process would take place while the air moves in a longitudinal direction.

That there is a minimum in the mixing ratio at all is perhaps surprising as the early ideas were that the mixing ratio should be constant with altitude above the tropopause. Indeed, an observation of increased mixing ratio at higher altitude was often explained as contamination¹⁰. But it is now

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thought that significant quantities of water vapour may be formed at higher altitudes by chemical and photochemical reactions which destroy methane¹², another gas that passes into the stratosphere through the tropical tropopause. In fact the results of these experiments will have a general applicability to the transfer of all tropospheric trace gases into the strato-

sphere¹³. For most of these the transfer is not limited by a physical property, such as the variation of saturation vapour pressure with temperature that limits the stratospheric water content, and if fresh tropospheric air is sampled above the tropopause in a region such as the western Pacific it should be possible to detect relatively short-lived tropospheric species.

Bright future for liquid electrolyte solar cells?

from A. Hamnett and S. Dennison

THE past decade has seen major efforts to develop photoelectrochemical devices that directly convert light to electricity. The devices are based on the junction between a semiconductor and a liquid electrolyte, but problems of corrosion and efficiency have bedevilled their successful development into a technology that could rival the more familiar solid-state p-n junction solar cells, where efficiencies of more than 20 per cent have been attained. The superiority of the solid-state p-n device may, however, appear a little less assured with a report in this issue of *Nature* by C. Grönet and N. Lewis of Stanford University (see p.733) who describe a photoelectrochemical cell (PEC) based on n-GaAs_{1-x}P_x in acetonitrile and using as redox couple the ferrocene-ferrocinium system. The cell has an operating efficiency of 13.2 per cent in natural sunlight, a figure considerably in excess of any previously reported from a non-aqueous cell.

The essential principles of the liquid-junction photovoltaic device can be understood with reference to the figure. If an n-type semiconductor is placed in a concentrated electrolyte and subjected to a positive bias, the region of the semiconductor near the surface becomes depleted of electrons. When light of sufficient energy is absorbed in this 'depletion layer', the electric field is able to separate the resultant photogenerated electron (in the upper or conduction band) from the positively charged vacancy or 'hole' left behind in the lower or valence band. The direction of the field is such as to drive the 'hole' to the surface, where it is transferred to the reduced form, R, of a suitable redox couple O/R. The electron, meanwhile, is transported around the external electrical circuit, performing work $e_0 V$, finally reducing O at the metal counter-electrode. No net chemical change occurs in the cell, but light energy $h\nu$ is converted to electrical work $e_0 V$. The figure makes it clear that there will be two limiting cases; the open-

circuit voltage V_{oc} , which is the maximum voltage obtained at zero current, will be approximately the difference between the potential corresponding to the conduction band edge and the redox potential of O/R. By contrast, the maximum current is obtained when the two electrodes are short-circuited. Between these two extremes, there will be a current-potential region where useful work can be obtained; owing to the spectral distribution of the solar spectrum, there will also be an optimal bandgap for the semiconductor which is ~ 1.4 eV, though bandgaps between 1.0 and 1.8 are likely to be effective.

Although the principles of this cell have been known for some years, it has proved extremely difficult to build practical devices based upon them. Four problems have arisen that have proved especially obdurate; of these, photocorrosion is the most serious and occurs when some of the photogenerated holes arriving at the surface become diverted from the desired reaction (conversion of R to O) and instead oxidize the surface of the semiconductor. To avoid this, a very fast rate constant for the oxidation of R is necessary, which minimizes the concentration of holes at the surface. A second related problem is that holes at the surface may also be diverted to electronic energy levels that are localized in the

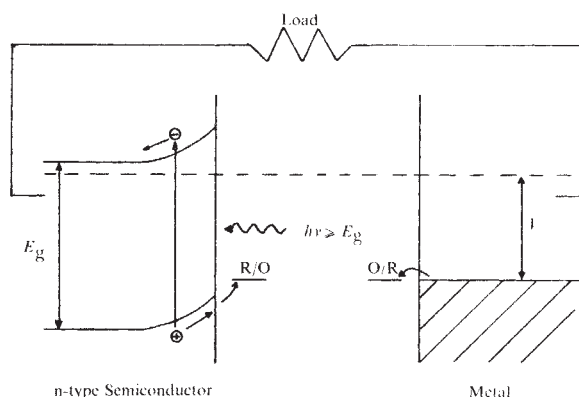
surface region — so-called 'surface states'. If these lie near the conduction band edge, they may in turn capture an electron from the conduction band, the net effect being the degradation of the light energy to heat. In aqueous solutions especially, surface states near the conduction band edge are very common and are thought to be due to protons. Much effort and ingenuity has gone into attempts to mitigate the deleterious effects of these states.

Closely related to this is the problem of grain-boundary recombination; ideally, the semiconductors used in such photovoltaic devices should be polycrystalline rather than single-crystal materials. From the economic standpoint, polycrystalline materials are enormously advantageous, but solid-state p-n junctions using them have proved extremely difficult to fabricate. This is because such junctions are made by taking a slice of n- or p-type material and diffusing into it, from the surface, impurity atoms that dope it locally in the opposite sense. Since diffusion down grain boundaries is far more rapid than diffusion in the bulk, it is prodigiously difficult to control the width of the junction in polycrystalline samples. No such fabrication problems arise in the liquid-junction photovoltaics described above, but recombination of carriers at the grain boundaries can cause a severe drop in operational efficiency.

The third problem is the kinetics of the O/R couple. We have seen that oxidation by holes must be rapid to compete with photocorrosion and surface-state capture processes, but additionally the kinetics of reduction of O to R must also be fast to reduce the overpotential at the metal cathode. In effect this means that the O/R couple must be highly electrochemically reversible.

Finally, strong chemisorption of the redox couple onto the electrode can occur. If both R and O strongly adsorb on the semiconductor surface, conversion of R to O will alter the charge balance at the interface. This in turn will reduce the electric field in the depletion layer, reducing the rate at which photogenerated holes can be

The design of the liquid-junction photovoltaic device. O/R is the redox couple.



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transported to the surface. A steady state is set up in which the change in the depletion layer charge is determined by the relative rates at which holes can be fed from O_{ads} to R in solution and the rate at which they can be supplied by the electrode. If the former rate is slow, the overall quantum efficiency will be very low.

In attempting to overcome these problems, a great deal of effort has been invested in pursuing a detailed understanding of the fundamental surface processes on semiconductors in contact with aqueous and non-aqueous electrolytes. In parallel with this work, novel methods of chemically modifying the surface to improve the quantum efficiency have been explored, and a large amount of phenomenological information gathered. Heller's group at Bell Laboratories has been especially active in this field, and has made considerable progress in reducing the effects of corrosion and surface-state recombination in cells based on aqueous electrolytes. The technological price has, however, been severe; water is a highly corrosive solvent, partly because of its excellent solvating properties, and complex measures for protecting the surface,

involving various chemisorbed metal ions, have had to be developed.

These technological difficulties have had a rather discouraging effect on aqueous photovoltaic work. In an effort to avoid some of the problems, several groups around the world have begun programmes of research into non-aqueous electrolytes. Initially, behaviour was found which suggested that chemisorption of the redox couples used was very common, and it was suggested that these cells would therefore have an intrinsic limitation to their efficiencies. However, it now appears from the work of Gronet and Lewis, reported in this issue, that no such intrinsic limitations exist. By using n-type $GaAs_{1-x}P_x$ electrodes and extremely straightforward cell design, they have obtained efficiencies of over 13 per cent. This is a remarkable result especially as, by the authors' admission, the cell is quite unoptimized. There can be little doubt that this result will lead to a considerable reassessment of liquid-junction photovoltaic devices and the obviously urgent task is now to investigate the use of polycrystalline electrodes to see whether the real advantages of such cells can be realized.

substrate of basement membrane components, all of which can improve the differentiated morphology of primary cultures (M. Prunieras, Fondation Rothschild; I. Mackenzie, University of Iowa).

In order to analyse normal differentiation or to define abnormalities in disease, it is essential to have good biochemical markers of keratinocyte differentiation; morphological criteria are not enough. Late in terminal differentiation an intracellular envelope of protein cross-linked by transglutaminase is formed. So far, two envelope precursors have been identified and solemnly named — involucrin (R. Rice, Harvard University) and keratolinin (not to be confused with keratohyalin) (L. Peterson, Oregon Health Sciences University). While it is still too early to say how many envelope precursors there are and how they are related, individually they provide good markers of terminal differentiation. Lectin binding by keratinocytes changes during differentiation, reflecting altered membrane glycosylation (P. Elias, VA Medical Center, San Francisco; F. Watt, Kennedy Institute). Such changes in keratinocyte surface properties might influence cell recognition and adhesion and could account for the sorting out of differentiating cells during stratification. Monoclonal antibodies to keratins reveal patterns of keratin synthesis characteristic of different stages of differentiation (T.-T. Sun, New York University). With elegant new STEM and linear mass density studies on keratin filament structure (P. Steinert, NCI) and with amino acid sequences of individual keratins obtained by gene cloning (E. Fuchs, University of Chicago; D. Roop, NCI), the biological significance of keratin heterogeneity may at last become clear.

Certain skin disorders, such as congenital ichthyosis, are characterized by distinct ultrastructural abnormalities which may continue to be expressed *in vitro* (I. Anton-Lamprecht, Institute for Ultrastructure Research, Heidelberg). However, good morphological criteria of disease are unusual in keratinocyte cultures, and many studies of potential clinical relevance are hampered by failure to exploit the known biochemical markers of normal terminal differentiation instead. This will surely change before long: it is exciting to learn, for example, that human cervical dysplasia is correlated with a decrease in involucrin synthesis, as this should be amenable to analysis in culture (R. Rice).

Keratinocyte culture club

from Fiona M. Watt

In recent years new techniques for the cultivation of epidermal cells (keratinocytes) have generated enormous interest among clinicians and biologists alike. Dermatologists are attracted by the possibility that keratinocytes from patients with skin disorders may behave abnormally *in vitro*. Given the clinical importance of squamous cell carcinomas, tumour biologists are discarding fibroblasts in favour of keratinocytes for many *in vitro* models of transformation and carcinogenesis. Keratinocytes appeal to the cell biologist too: as a system for studying terminal differentiation and population kinetics; for investigating basement membrane synthesis and the structure and function of keratin filaments. Scientists with these diverse interests gathered recently at a symposium on keratinocyte biology* which turned out to be lively and thought-provoking.

Methods of cultivating keratinocytes seem to proliferate more rapidly than the cells themselves, but generally fall into two categories: first, relatively simple systems for serial passage of cells, in which some of the properties of intact epidermis are retained; second, more elaborate attempts to reconstruct true epidermis in primary culture by approximating a normal

epidermal environment. Of the first group, the most successful is undoubtedly that of Rheinwald and Green (*Cell* 6, 331; 1975), in which human keratinocytes can be grown for many generations on a feeder layer of 3T3 cells. There have been several attempts to eliminate the requirement for feeder cells and a new chemically defined medium for human keratinocytes seems quite promising (S. Boyce, University of Colorado). It is harder to persuade mouse keratinocytes to proliferate *in vitro*, and consequently most cultures are seeded at high density. A low level of calcium ions (< 0.1 mM) enhances proliferation of newborn mouse epidermal cells (H. Hennings, NCI), but as this prevents stratification, normal terminal differentiation, in which a steady state is achieved between basal cell division, suprabasal differentiation and cell shedding, is not observed.

The histological appearance of cultured epidermis can be improved by growth on a 'dermal equivalent' — a collagen gel containing fibroblasts (E. Bell, MIT). Further attempts to mimic the normal environment of epidermal cells include culturing at an air-liquid interface, feeding cells from below and providing a growth

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Corrigendum

In the *News and Views* article 'Transformation and gene organization in *Drosophila*' (*Nature* 300, 15; 1982), the work on cell division mutants (penultimate paragraph) was attributed solely to B. Baker's *La Jolla* laboratory. Since its inception, this research has in fact been done in collaboration with the laboratory of M. Gatti (Rome).

*'Biology of the Keratinocyte In Vitro', 32nd Annual Symposium on the Biology of Skin, was held at Gleneden Beach, Oregon on 10-14 October 1982.

REVIEW ARTICLE

Changes in the solar constant and climatic effects

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Spacecraft measurements have established that the total radiative output of the Sun varies at the 0.1–0.3% level. The observed fluctuations are well modelled as radiative deficits proportional to the area of the solar disk covered by sunspots. Historical records of projected sunspot areas allow an accurate reconstruction of these short-term fluctuations over the past century. Such changes can be expected to perturb the terrestrial surface temperature by a fraction of a degree centigrade and probable evidence of this solar-induced signal has been found. The effect, though important in terms of understanding the climate system, is too small to be significant in practical weather or climate predictions.

RADIATION from the Sun is the dominant source of energy input to the Earth's atmosphere. Solar energy, coupled with the rotational inertia of the spinning planet, drive the circulation patterns of oceans and air that are the basis for all the phenomena known as weather and climate. Regional weather patterns may result from local perturbations of geographical, orographic, or other origins, including anthropogenic ones, but all of these processes depend on a fundamental flux of solar energy. The equilibrium temperature of the Earth's surface and seas is ultimately determined by a balance between solar radiation absorbed by the Earth, primarily at visible and near IR wavelengths, and long-wave radiation that is re-emitted to space. Should the total solar input vary as a persistent trend, the surface temperature of the Earth will in time respond in a direct and predictable fashion: global cooling in response to decreased solar radiation, and warming following an increase. The diurnal variation of cold nights and warmer days, and seasonal patterns from winter to summer attest to the role of insolation as the principal temperature control.

Such reasoning has prompted innumerable attempts at identifying the signature of solar variability in climate records. What is surprising is how difficult it has been to find any unequivocal evidence of any significant forcing by known solar variability^{1,2}. Meadows³ has traced the spotted and often confused history of such attempts, most of which were statistical tests of supposed connections between observed solar activity and various meteorological parameters. He adopted an earlier estimate⁴ that "Solar activity does not produce a significant variation [upper limit of 0.1%] in the value of the solar constant". With this exclusion, any significant Sun-weather effects depended on indirect or 'trigger' mechanisms, which might result, for example, from perturbations in the energetically weak solar wind on the Earth's upper atmosphere and possible subsequent tropospheric response. Meadows concluded that: "If [an acceptable mechanism] could only be defined, the whole controversial field might be rapidly translated into the realms of high respectability".

We discuss here recent evidence for measured variations in the solar luminosity which provide a direct and simple mechanism for associated climatic change. Such variations, though small in amplitude, constitute a significant breakthrough in solar-terrestrial physics: for the first time a form of solar variability has been identified that is sufficiently energetic to alter directly the basic radiation balance of the lower atmosphere. Moreover, a wholly consistent response in surface temperature has already been (independently) identified⁵, lending credence to the reality of the connection. The resultant changes in surface temperature

are very small—no more than a few tenths of 1 °C. But the existence of a probable response opens the door to the possibility of larger effects, due to longer and more important changes in the bulk radiative output of the Sun.

Observational evidence for solar constant changes

Precision measurements of the total solar flux, or 'solar constant', S , have long been desired; they have become possible on a continuous basis only in the present decade with sophisticated radiometers in Earth orbit. Langley had foreseen the need to rise above the atmosphere 80 yr ago when he called for the establishment of an elevated solar observatory⁶: "The Earth's temperature and the life of its inhabitants, both animal and vegetable, depend on the solar radiation. Yet we confess that even at this late day we do not know, with any certainty, what the total amount of solar radiation is, whether it is constant or variable, or what effect upon terrestrial life and temperature a given change in it would produce. Our ignorance of these fundamental things is largely, though not wholly, due to the variability of our own atmosphere, which prevents us from studying that of the Sun".

In 1881, Langley had attempted a precision measurement of the solar constant from the top of Mt Whitney, 14,495 ft. elevation. Subsequently, he and Abbot for many years tried to measure the solar constant from mountain tops but their measurements were still limited by uncertainties in atmospheric transmission to about $\pm 1\%$ ⁷. In 1980, near the peak of the

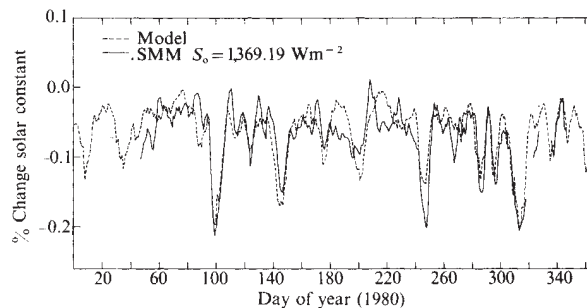


Fig. 1 Comparison of the model results to the SMM measurements for 1980. A quiet Sun solar irradiance of $1,369.2 \text{ W m}^{-2}$ is assumed for the SMM measurements and per cent deviations are from this value. Most of the differences between the model and measurements can be attributed to random errors in the measured sunspot areas.

solar activity cycle, the SMM spacecraft succeeded in accumulating nine months of continuous measurements of the solar constant with an unprecedented internal precision of 0.01%⁸. In these data was clear evidence of changes at the level of 0.1–0.3%, about an order of magnitude lower than the threshold of previous, ground-based measurements. A similar radiometer on the Nimbus 7 spacecraft^{9,10} confirmed that these excursions, lasting 1 to 2 weeks, were depletions in the total output of the Sun. With these data the possibility of a rigorously constant S was unambiguously disproven for the first time.

Theoretical understanding of solar constant changes

The continuous solar constant measurements accumulated by the SMM in 1980 are shown in Fig. 1, as daily averages. Error bars are approximately the width of the line used to trace the record. It is obvious that the variations of S seen by the SMM are not those of a white noise spectrum, but are well approximated by a steady background with minor rises and major, superposed dips of varying amplitude. The time series of Fig. 1 suggests a process by which S is depleted over time scales of days to weeks without a compensating emission process. Moreover, initial analysis of the SMM data established the fact that the major dips were always coincident with the passage of large sunspot groups across the solar disk.

Sunspots are localized regions on the solar surface which appear dark in relation to the background solar photosphere (Fig. 2). The umbra, or dark central region of a sunspot, has a temperature of about 4,000 K, some 1,800 K or 30% cooler than the adjacent photosphere. A sunspot penumbra has a typical temperature of about 5,400 K.

Sunspots are regions of intense, concentrated magnetic field. These strong magnetic fields inhibit the local, vertical, convective transport of energy, leading to a lower, local radiative flux and a corresponding reduction in local temperature. It has long been suspected that the presence of sunspots, which are known to follow an 11-yr recurrence cycle, could modulate the Sun's total radiative output. In serious question, however, has been whether solar activity would decrease¹¹ or increase^{12,13} the total solar flux, because dark sunspots are always accompanied by bright (hotter) facular regions in the photosphere and chromosphere. A third and equally tenable hypothesis was that a detailed balance in S would ensue between sunspot depletion and facular emission, resulting in a rigorously invariant solar constant. The SMM and Nimbus measurements establish that

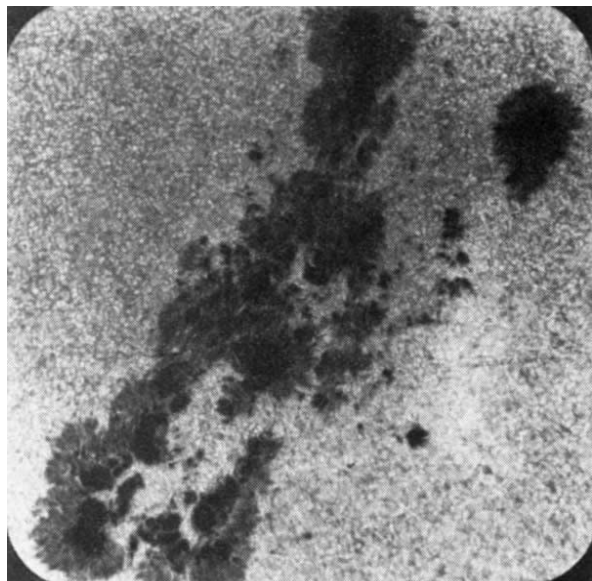


Fig. 2 A large sunspot group showing dark umbral and intermediate penumbral regions contrasted against the brighter surrounding photosphere. (Sacramento Peak Observatory, Association of Universities for Research in Astronomy, Inc.)

only a small fraction of the energy blocked by sunspots is balanced by immediate enhanced emission from bright features such as faculae and plages^{8,14–16}. On time scales of months or years, there is no detailed balance. As we shall see, this fact has practical consequences for the effect of solar variability on the Earth's temperature.

The basis for these important conclusions is the success of simple, blocking models in replicating the observed pattern of fluctuations in S that were observed with the SMM and Nimbus spacecraft (Fig. 1) using daily, observed values of projected areas and positions of sunspots made by ground stations. The symmetric, gaussian shapes of the major dips are readily explained as the result of spherical projection: a sunspot of fixed size will block more of the apparent area of the solar disk as solar rotation carries it towards the Sun's central meridian. Furthermore, the magnitude of the observed depletions in S are well duplicated by measured sunspot areas and canonical values of umbral and penumbral contrasts.

Schatten *et al.*^{17,18} have endeavoured to attribute considerably more of the observed fluctuations in S as due to facular brightening, thereby implying a detailed balance in the total solar flux. This divergent interpretation is challenged by other, independent studies of the same spacecraft data^{8,14,15,16,19–21}. As explained elsewhere^{22,23}, the discrepancy may be due to the failure of studies implying detailed balance to make use of actual, daily observations of sunspot and facular areas or to utilize the full SMM solar constant record. When this is done a better fit and a more accurate interpretation is obtained. The difference in terms of the storage of radiative flux within the Sun and in terms of potential climate effects is fundamental.

The model for sunspot blocking used in Fig. 1 is based on daily measured positions and areas of sunspots and on standard umbral, penumbral and facular contrasts of -0.75 , -0.25 and $+0.03$ respectively¹⁴. Also included is the well-known effect of limb-darkening. The brightness of the apparent disk of the Sun decreases towards the edge, or limb; as a consequence sunspots near the limb have lower contrast relative to the background Sun than they do near the centre of the disk. The amount of irradiance blocked by a sunspot therefore decreases as it moves away from the apparent centre line, seen from the Earth, due to a combination of geometrical foreshortening and limb darkening. The model used in Fig. 1 also assumes that the blocked radiation is never released. In time it must be, but with the data now available it is possible only to conclude that the blocked energy does not reappear in recognized form for time scales of at least months, and probably a year. Continued measurements should help to refine the estimate of effective storage time. The notion of a frustrated Sun, with stored, and gradually released energy from remembered sunspots is not an altogether unreasonable one. Blocked, radiative energy can be stored in mechanical form in the lower convection zone with a relaxation time of up to 10^5 yr, (refs 24–26) during which time it will only slowly seep out. For purposes of comparisons with SMM or Nimbus data a storage time of this length is almost infinity.

The remarkable fit of SMM observations with simple, sunspot blocking models allows us to take two further steps. We can now predict the short-term excursions in S (should climatologists want them) a few days in advance, based on easily obtained measurements of sunspot areas and positions and the known rotational properties of the Sun. By the same technique we can also reconstruct the past history of daily fluctuations in S from archived sunspot data. Precise records of sunspot areas and positions exist for more than 100 yr. Figure 3 shows reconstructed, daily records of S for four sample years as they were generated from historical sunspot records¹⁴. The 108-yr reconstruction shows the expected signature of the 11-yr sunspot cycle, with greater accumulated depletion during years of enhanced sunspot numbers.

These predictions and reconstructions tell all we know with certainty of solar constant excursions. But there may well be more to the story in the form of longer, possibly independent

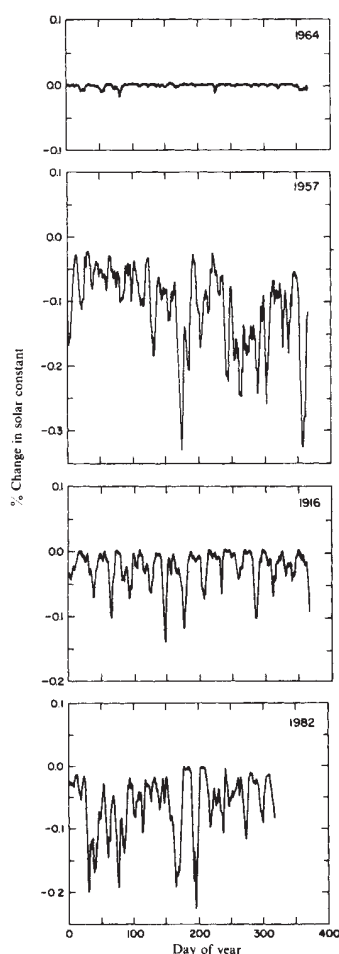


Fig. 3 Daily solar constant values reconstructed from sunspot and facular area records for sample years of low activity, high activity and recurrent, moderate activity with a similar reconstruction for 1982, to 14 November.

trends. Such trends, if they exist, could be of greater climatic impact, through persistence, than the short-term sunspot blocking effects noted above. The existence of one such possible trend, an apparent decrease in S of $\sim 0.02\text{--}0.04\%$ yr^{-1} , has already been noted in the Nimbus²⁷ and SMM²⁸ records. The model shown in Fig. 1 would predict a downward trend of about this amount in the 1979–82 period due to an averaged increase in sunspot areas during this time, even though sunspot numbers were simultaneously decreasing. It is probably too early, however, to determine whether the measured trend is real, and, if real, how much of it is due to accumulated sunspot blocking or some other effect.

We may sum up the recent discoveries concerning the solar constant as follows:

- (1) The solar constant varies.
- (2) The fluctuations are almost wholly explained by the simple mechanism of sunspot blocking, by which S is reduced in direct proportion to the fraction of the apparent solar disk that is covered by sunspots.
- (3) Excursions in S during high solar activity are at most 0.5%, and more generally 0.1% or less, maximizing in each case when large sunspot groups are near the central meridian.
- (4) Excursions of this nature persist throughout the period of visibility of the spot—for 14 days or less.
- (5) The radiation blocked by sunspots does not immediately reappear where it can be seen or sensed; it is in some way stored within the Sun, to seep out much more slowly as an integrated increase in radiative flux.
- (6) Photospheric and chromospheric faculae (surface regions brighter than the background disk) play at most a minor role in modulating the instantaneous value of S .

Evidence for a solar cycle effect in temperatures

A possible, 11-yr signal due to sunspot effects has long been sought in both regional and globally-averaged surface temperature records^{29–34}—a search that has origins in ancient weather lore³⁵ and in literature^{36,37}. The lack of any clear success in these attempts almost certainly implies that any globally distributed, coherent variation due to sunspot-cycle forcing must have a small amplitude. Still possible, however, are enhanced, regional or seasonal responses in areas of extended land mass and lower thermal inertia. Currie⁵ has found statistically significant evidence for such a variation of annual-averaged surface temperature in 53 stations in the northeastern sector of North America, based on recent analyses of 80 yr of surface data. The variation found by Currie has a mean amplitude of about 0.18°C ; it was absent or undetectable in other regions of North America for which extended records exist. The mean period of the temperature variation was 10.5–10.7 yr, which may be compared with the mean sunspot blocking period shown in Fig. 4 of ~ 10.6 yr. Currie found that the air temperature lagged sunspot numbers by 5.7 ± 0.7 yr in the sense that temperatures tended to be lower at maxima in the 11-yr sunspot cycle; this is almost precisely what would be expected were the temperature effect due to sunspot blocking, with a reduced solar constant at times of high sunspot numbers. The apparent confirmation of a suspected regional, solar-climate effect is intriguing; before it can be accepted, however, it should be tested on other continents and with realistic climate models. But a small solar constant signal, restricted to an inland region geographically removed from the thermal inertia of the oceans and downwind of a major mountain chain is not unreasonable.

We have found further possible evidence in the same geographical region by examining the occurrence of temperature extrema in a data set³⁸ of mean monthly temperatures for the 48 contiguous US states over the time span 1900–80. The occurrence of a monthly high temperature record was a factor of 3 more frequent during the three years of minimal sunspot blocking than during the three years of maximum sunspot blocking. The same effect is found in the remaining regions of the continental US although with a slightly weaker signal. This factor of three greater probability of finding temperature maxima at minimum solar activity is a statistically significant result, with only a 1% probability of occurring by chance. The corresponding occurrence of record low temperatures during maximum sunspot blocking has a much weaker mirror relationship: record low temperatures were 30% more frequent in maximum than in minimum sunspot blocking years, although there is a 20% probability of this having occurred by chance.

Theoretically expected temperature response

What global surface temperature response should we expect from the reconstructed solar constant forcing? A reasonable answer is shown in Fig. 4, from a simple global climate model that assumes energy balance between incoming solar radiation and outgoing IR^{39,40}. The model includes terms for the thermal inertia of the oceans: a mixing layer (upper level equivalent 87 m of water) with a thermal response scale of 5–10 yr, and a deep ocean with a volume 20 times larger. The effect of a

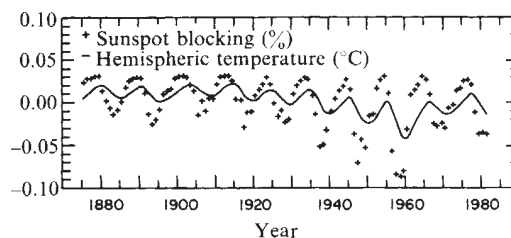


Fig. 4 The yearly mean deviations in solar irradiance indicated by the sunspot blocking model for 1874–1981 with expected hemispheric temperature variations as calculated using a simple energy balance climate model.

short-term change in insolation will be moderated by the large thermal inertia of the strongly-interacting surface regions (atmosphere, land surface and ocean mixed layer) of the Earth. The degree of attenuation will depend on the frequency and persistence of the forcing signal. A cyclic perturbation in insolation with a period of 11 yr will induce a surface temperature response that is a factor of 2–3 smaller than the equilibrium response to the same forcing. The equilibrium response of surface temperature to solar constant changes is generally estimated at 0.1°C for a 0.1% radiative perturbation⁴¹. Peak-to-peak variations in solar forcing of 0.12% on an 11-yr cycle derived from extrapolated sunspot blocking models will lead to a similar cycle in mean global temperature with an amplitude of only 0.06°C . Possible feedback mechanisms make this estimate uncertain by a factor of at least 2, but more detailed calculations of atmospheric circulation tend to support the energy balance arguments given above.

The atmosphere itself has a thermal inertia time scale far shorter than 1 yr (as evidenced by diurnal and seasonal variations). Therefore an inland region that is isolated from ocean temperature moderation can react to solar forcing in a manner more nearly that of the theoretical equilibrium response. Regions at high latitude will also experience larger responses due to the positive feedback of snow-albedo in which decreased solar radiation leads to greater snow cover, hence higher albedo and an even lower surface temperature. Currie's finding⁵ of a weak, 11-yr cycle in inland North American air temperatures is therefore consistent in period, phase and amplitude with that expected theoretically from reconstructed sunspot blocking¹⁴.

One can also test the compiled record of annual mean Northern Hemisphere temperature⁴² for evidence of the same effect. We have done so and find that the mean hemispheric temperature during 1881–1981 was on average 0.036°C cooler in the 3 yr of peak sunspot blocking as compared with the adjacent 3 yr of minimum solar activity. The reconstructed solar constant for the same period exhibits mean peak-to-peak variations of 0.06%; hence the observed temperature change is almost exactly what one should expect for a hemispheric average, based on simple climate models. Unfortunately, as is so often the case in solar-climate studies, the finding just cited is not statistically significant—about 50% of the time, given the statistics of the population sample, the same correspondence could occur from mere chance.

The response of surface temperature to forcing by sunspot blocking can be summarized as follows:

- (1) On a hemispheric or global scale surface temperature responds to the 11-yr sunspot cycle, with an amplitude of 0.036°C . However, this variation, although in accord with predictions of simple climate models, is not statistically significant.
- (2) Station records for the past 80 yr in the north-east sector of the US establish that the mean surface temperature responds to solar, 11-yr forcing with an amplitude of a few tenths of 1°C .
- (3) A significant variation in the temporal distribution of record high monthly mean temperatures occurs in the same geographical region cited above, in response to solar forcing. The number of record low monthly mean temperatures also responds in a manner consistent with solar forcing, but not in a statistically significant way.

The phase of the relationship between sunspots and the solar constant, described above, is precisely opposite that which has been proposed to link longer-term changes in the envelope of solar activity with century-scale changes in climate⁴³. The possible association of the Maunder and Spörer minima of solar activity, lasting 70 and 130 yr, with coincident cold periods of the Little Ice Age has been interpreted, for example, as the result of a postulated drop of about 1% in the solar constant⁴⁴. The simple extrapolation of the mechanism of sunspot blocking to century-scale changes in the envelope of the activity cycle would induce an apparent secular modulation of at most 0.1%, with an increased solar constant at times of suppressed activity, such as the Maunder Minimum. Whether such an extrapolation

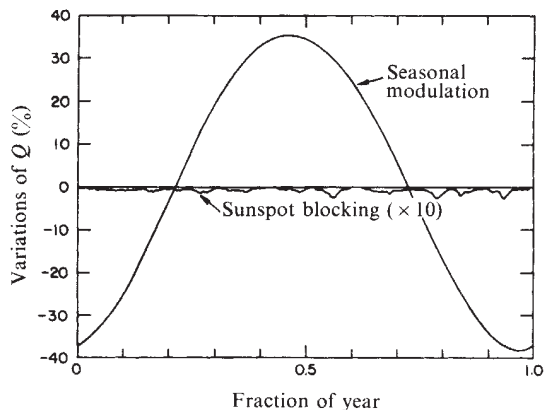


Fig. 5 Variation of daily solar irradiance (represented by Q) about the yearly mean due to seasonal effects in per cent deviation from a hemispheric average. The solar constant reconstruction for 1981 is shown multiplied by a factor of 10 for comparison.

is justified is another question. It is altogether possible that solar processes other than accumulated sunspot blocking act to modulate the solar constant in the longer term, and that such processes could oppose, in sense, the higher frequency modulation due to the 11-yr sunspot cycle. Such long-term changes are, of course, wholly speculative, since precision measurements of the solar constant span no more than 4 yr.

Importance of climatic response

As shown above, both observation and theory seem to support the case for a small but direct surface air temperature response to measured and extrapolated solar constant fluctuations that is within a factor of 2 of the expected change in the solar luminosity.

These conclusions suggest several practical questions: (1) Is the apparent temperature response detectable as a perceptible weather effect? That is, can the average person sense a solar constant perturbation? (2) Will the anticipated climatic change be significant in practical or economic terms? (3) Can identifying the cause of climatic change at this level lead to better weather prediction?

Considering that the amplitude of the solar-induced mean temperature signal is but a few tenths of 1°C , the answer to all of the above questions must surely be negative. A surface temperature effect of this magnitude is truly insignificant and for practical purposes imperceptible in the presence of typical diurnal extremes of 10° and a seasonal range of at least 30° . It is hard to see how a cyclic variation of a few tenths of a degree in the mean could have either predictive or economic import.

However, a factor of 3 enhancement in the frequency of temperature maxima occurring over a sunspot cycle could yield a marginally positive answer to all three of our pragmatic questions. People are much more likely to notice and remember unusual climate extremes. In economic terms it is again the extremes which cause dislocations. A factor of 3 change in the probability of occurrence of extrema in temperature is also of possible though limited predictive power.

Another way of gauging the climatic importance of a small perturbation to the solar constant is to compare it with the seasonal cycle of insolation. In Fig. 5 we compare the intra-annual variation of daily sunlight received over the Northern Hemisphere with the variation of S as reconstructed from sunspot blocking for 1981 (the latter is multiplied by 10 to make it visible). As one can see the interannual variation due to seasonal effects easily overwhelms that due to sunspot blocking. As another test, if we define the onset of spring to be 21 March in a year with 'normal' solar irradiance, how much will anticipated insolation changes due to sunspot blocking shift the effective first day of spring? In a year with high sunspot blocking (such as 1981 or 1982) the integrated irradiation reaches that of a normal spring about 0.2 days late. One effect of a sunspot-blocked solar constant could thus be the delay of the onset of

spring by a few hours. By comparison, the meteorologically defined onset of spring, the date of last severe frost, has a typical variation of about 21 days and extreme differences of about 90 days in mid-latitude regions. Hence any shift due to the solar constant sunspot blocking is unimportant compared with typical variations, just as any induced change of mean surface temperature is also small compared with typical variations. This could be qualified, perhaps, by the fact that the mean sunspot blocking effect extends over a few years, thus allowing seasonal variations to be averaged over, making it slightly easier to detect the small effect.

Discussion

Solar constant variations of at most 0.5% for a few days and ~0.1% for a few years are now known to exist, but their expected effects on surface temperature are by any measure minor. Modelled or measured climate responses are insignificant compared with normally occurring changes and to apparently random variations in temperature. Thus it seems wholly unjustified to attribute the coincidence of a harsh winter such as that of the past year in some regions to a direct effect of a coincident solar constant deficit²⁸.

On the other hand, the importance of climate responses which might result from solar constant variations should not be dismissed. The identification of a simple, external forcing function such as a variable solar constant, which induces detectable changes of mean temperature, however slight, may provide a practical test of climate sensitivity on regional scales. Climate sensitivity verification is crucial to understanding such problems

as the impact of anthropogenic carbon dioxide increases and land use changes. In a world where the demand for food is ever closer to the available supply, it may be true that any predictable fluctuation in climate will in time become important.

Perhaps the most important conclusion of the variations now established in the solar constant is that the Sun is indeed variable in its most fundamental output and that plausible responses of surface temperature have now been at least tentatively identified. Thus to increase our understanding of solar variability, we must continue the precision monitoring of the outputs of the Sun.

The demonstrated connection between changes in the solar constant and surface temperature exemplifies the kind of effort needed to redirect the study of solar-terrestrial relationships. Precision, spaceborne measurements of the solar output have enabled us to identify, and delimit, at least on time scales of days to years, a real climatic effect. Important questions remain unanswered. What are the detectable impacts of measured changes in solar short-wave radiation, or in the variable, particle inputs to the upper atmosphere? Do 'trigger' mechanisms have a role in amplifying the effects of variations in *S* or in other, weaker outputs of the Sun? Is the solar constant variable, perhaps in different or opposing ways, on longer time scales? New attempts at answering these and other such questions must surely focus not on statistical correlations but on mechanisms, modelling, and above all, measurements. Until we know how and in what ways the Sun varies we can only guess the possible terrestrial consequences.

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ARTICLES

The 5-min oscillations of the Sun are incompatible with a rapidly-rotating core

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Hill's suggestion that Einstein's general relativity may be invalid requires a rapidly spinning solar core. The triplet structure observed in the solar 5-minute power spectrum seems incompatible with this rotating core. But the triplet structure can be accounted for as the effect of a magnetically distorted core rotating with a ~12.5-day period.

FROM a study of the fine structure of solar oscillations Hill^{1,2} has concluded that the Sun's core is rotating with a period of ~4 days. From a consideration of the effect of the resulting J_2 on Mercury's motion he concludes that

Einstein's general relativity may be invalid¹. From the same data Gough³ agrees that the Sun contains a rapidly rotating core, but he disagrees with the implication *vis-à-vis* Einstein's theory.

An even shorter rotational period (1 to ~2 days) was previously obtained from solar oblateness measurements (a P_2 distortion) made in 1966^{4,5}. It was later found that a substantially better fit to the data could be obtained from an alternative hypothesis, that the solar distortion is not axially symmetric and that the distorted solar figure (the gravitational potential surface) rotates rigidly over the solar surface, as a wave, with a 12.38 ± 0.10 -day period (sidereal)^{6,7}. It has been suggested that the 12.38-day period represents the rotational period of the core and that the distorted gravitational potential is due to a strong magnetic field buried in the solar core⁷⁻⁹.

Clearly the 4-day period of Hill *et al.* and Gough, and this 12.4-day core rotation period are incompatible. Which if either is correct? The triplet structure discovered by Claverie, *et al.*¹⁰ in the $l=1$ '5-min' oscillations may yield the answer (see Fig. 1).

This triplet structure was first interpreted as rotational splitting of the $m=1, 0, -1$ components of the $l=1$ modes, the effect of advection of the modes¹⁰. With this interpretation the splitting is too small to be compatible with the ~4-day period and with the rotational curves given by Gough³. It yields a core rotation period of ~12 days for a rigidly rotating core with a radius of 0.6.

Isaak¹¹ has noted that owing to the symmetry of the observation, an average over the solar disk, the central ($m=0$) component of the triplet (Fig. 1) should be missing for a solar axis perpendicular to the line-of-sight. For the actual solar axis tilt ~6° the $m=0$ component should be very weak, with an intensity of 0.005. Isaak has suggested that the axial symmetry of the sun is broken by an off-axis magnetic field in the solar core rotating with a 12-day period and that the ' $m=0$ ' component is present because it contains an admixture of $|m|=1$ induced by the magnetic field.

Gough¹² has noted that the same symmetry requirement would yield a triplet structure for rotational splitting of the $l=2$ oscillations (without $m=\pm 1$ components), but he considers an $l=2$ origin of the triplet implausible. With an $l=2$ origin and the splittings given above (Fig. 1), the core of the Sun must rotate slower than the surface. This is very unlikely. Furthermore, Gough¹² considers the $l=1$ identification to be statistically robust.

The fine structure of the $l=1$ '5-min' oscillation seems to be incompatible with a purely rotational splitting induced by a rapidly rotating core. Is it compatible with the type of splitting to be expected from a magnetized solar core rotating rigidly with a 12-day period? Gough¹² has already examined this

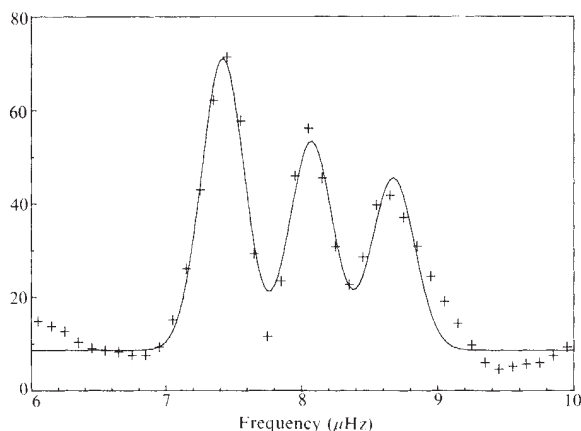


Fig. 1 Fine structure of $l=1$ modes of '5-min' oscillations. The data points were taken from Fig. 2b in ref. 10. (The frequency origin is arbitrarily chosen.) The curve is an eight-parameter fit of the sum of a constant and three gaussians having a common width. The three frequencies obtained are: $\nu_1 = 7.419 \pm 0.009$, $\nu_2 = 8.071 \pm 0.012$ and $\nu_3 = 8.676 \pm 0.015$ μHz . The two frequency splittings differ by only 1.6σ . The mean splitting is 0.6285 ± 0.0087 μHz . The errors are only formal standard deviations. The gaussian width (σ) is 0.158 μHz .

Table 1 Matrices $R(\alpha)$, $P(\beta)$ and T

$\begin{bmatrix} \cos \alpha & -\sin \alpha & 0 \\ \sin \alpha & \cos \alpha & 0 \\ 0 & 0 & 1 \end{bmatrix}$	$\begin{bmatrix} \cos \beta & 0 & \sin \beta \\ 0 & 1 & 0 \\ -\sin \beta & 0 & \cos \beta \end{bmatrix}$	$\begin{bmatrix} 1 & -1 & i & 0 \\ \frac{1}{\sqrt{2}} & 0 & 0 & \sqrt{2} \\ \sqrt{2} & 1 & i & 0 \end{bmatrix}$
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Elements of the rotation matrices $R(\alpha)$ (z axis), $P(\beta)$ (y axis) and the transformation matrix T (for change of basis from W_n to $Y_{1,m}$).

question making several simplifying assumptions and found that he could not obtain a satisfactory account of the splitting using this solar model.

One of Gough's assumptions can be questioned. He correctly notes that both the perturbation of the wave by the magnetically distorted core and the rotationally induced advection of the propagating wave are small effects and can be linearly superposed. He consequently assumes that the normal mode structure can be calculated by first neglecting the effect of solar rotation (including only the magnetically induced perturbation) and then adding the advection to this normal mode.

It is shown here that this assumption seems to be untenable. When the solar core is rotating more rapidly than the surface it is necessary to include the effect of advection before the normal oscillatory modes are calculated. Both the structure of the normal modes and their frequency perturbations can be strongly affected by advection.

Normal oscillatory modes

The normal modes for $l=1$ are to be calculated assuming that the solar core (of radius r_c) is distorted by an internal magnetic field and is rotating rigidly with an angular velocity Ω_c . It is assumed that outside the core ($r_c < r < r_0$) $\Omega(r)$ is a function of r only. To avoid the complications arising in the time dependence of the magnetic perturbation we follow Gough's technique of employing a coordinate frame fixed to the core. The small Coriolis effect is neglected.

In this coordinate frame the solar core is static whereas the outer shell is rotating in a steady state. In the absence of dissipation the wave equation is time-reflection invariant in the core.

Expressed in terms of the radial displacement of the fluid ξ , the $l=1$ unperturbed normal modes (complex) are conveniently separated into ingoing and outgoing waves, ξ_- and ξ_+

$$\xi_- = \sum_n a_n W_n(\theta, \phi) f(r) \exp(-i\omega t) \quad (1)$$

$$\xi_+ = \sum_n b_n W_n(\theta, \phi) f^*(r) \exp(-i\omega t) \quad (2)$$

(By an unperturbed normal mode is meant a normal oscillatory mode for a non-rotating Sun with an undistorted solar core.)

Near the centre of the Sun the complex radial function $f(r)$ has the form of the derivative of a spherical Hankel function

$$f(r) = -i \frac{d^2}{dr^2} (\exp(-ikr)/k^3 r) \quad (3)$$

Elsewhere it depends on details of the solar model. $f(r) + f^*(r)$ is singularity free at $r=0$. For the unperturbed normal mode the constant $b_n = a_n$. For the perturbed wave the (complex) a_n and b_n are functions of r .

The waves are expanded in real surface harmonics of $l=1$ (W_n) which are in turn expressed in terms of the usual (normalized) complex spherical harmonics $Y_{l,m} \sim P_l^{|m|} e^{im\phi}$

$$\begin{aligned} W_1 &= \frac{1}{\sqrt{2}} (-Y_{1,1} + Y_{1,-1}) \\ W_2 &= \frac{i}{\sqrt{2}} (Y_{1,1} + Y_{1,-1}) \\ W_3 &= Y_{1,0} \end{aligned} \quad (4)$$

The real surface harmonics, W_n , are required to incorporate

conveniently the time reflection invariance of the solar core.

The calculation of the three normal modes for $l=1$ and a fixed radial order is carried out in four steps:

(1) We start with the ingoing wave evaluated at the surface of the core and characterized by the three wave amplitudes a_1, a_2, a_3 . The ingoing wave passes through the core and reappears at the surface as an outgoing wave characterized by b_1, b_2, b_3 . Owing to the influence of the core magnetic field, the velocity of sound is not uniform on spherical surfaces and the wave propagating through the core undergoes an angle dependent phase shift. The outgoing amplitudes b_1, b_2, b_3 describe the $l=1$ part of this phase shift. The a s and b s are linearly related. In matrix notation

$$b = Sa \quad (5)$$

where a and b are column vectors containing their three elements of the order (1) ... (3). The so-called scattering matrix S is both unitary and symmetric. In the absence of the core distortion $S = I$ is the identity matrix.

(2) After leaving the core, the outgoing wave passes through the outer shell, is reflected at the solar surface and returns as an ingoing wave to the surface of the core where it has an amplitude a' . In this transit through the rotating shell the wave is rotated through a (negative) angle $\Delta\phi$ given by

$$\Delta\phi = 2 \int_{r_c}^{r_o} (\Omega(r) - \Omega_c) dr/c \quad (6)$$

where c is the sound velocity.

$$a' = R(\Delta\phi) \cdot b \quad (7)$$

where $R(\Delta\phi)$ is the rotation matrix which rotates b by $\Delta\phi$ into the orientation of a' (see Table 1).

(3) To represent a normal mode (a') must be identical with (a) except for the phase shift factor $\lambda = \exp(-i\Delta\omega\tau)$ due to shift $\Delta\omega$ in the angular frequency. Here $\tau \approx 7,400$ s is the round trip transit time of the wave. Combining equations (5) and (7) and setting $a' = \lambda a$ yields the eigenvalue equation

$$(RS)a_i = \lambda_i a_i \quad (8)$$

The matrix (RS) is unitary and its three eigenvalues λ_i are

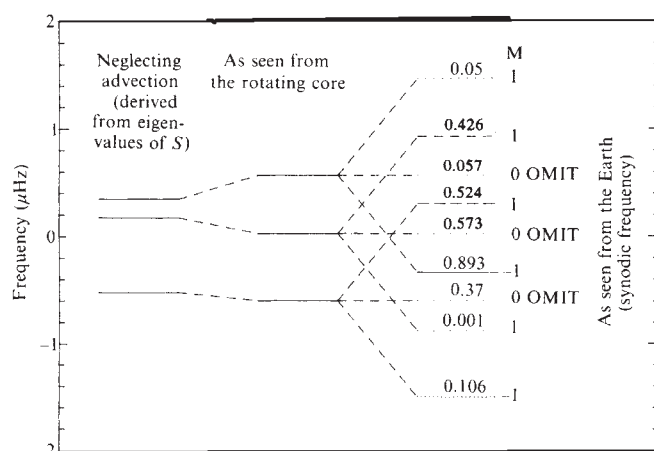


Fig. 2 The computed fine structure of the $l=1$ component of the '5-min' oscillation of the Sun. The three phase angles of the scattering matrix, $s_1, s_2, s_3 = -0.0162, -0.0081$ and $+0.0242$, have ratios that differ only slightly ($\sim 1\sigma$) from the ratios of the three principal moments of inertia of the solar core. The mode advection angle is $\Delta\phi = -0.0167$ and the core orientation angles are $\alpha = 1.59, \beta = 0.787$ rad. The plotted frequencies are: (1) $\Delta\nu_i = -s_i/2\pi\tau$, for which the effect of advection is neglected ($\tau = 7,400$ s); (2) $\Delta\nu_i = \Delta\omega_i/2\pi$, equation (8), where the effect of advection $\Delta\phi$ is included but the mode is observed from the rotating core; (3) the same as (2) above but as viewed from the Earth (synodic frequencies) $\Delta\nu_{im} = (\Delta\omega_i + m\Omega_c)/2\pi$ with the spherical harmonic orders m and the line intensities labelled. The intensities are chosen to sum to unity for each mode. $\Omega_c/2\pi = 1/12.81 \text{ day}^{-1} = 0.903 \mu\text{Hz}$.

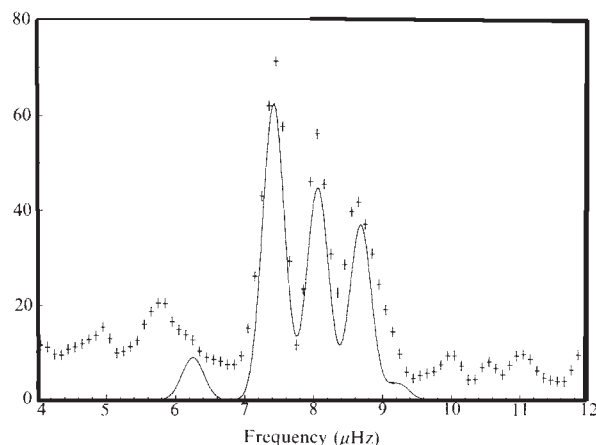


Fig. 3 Plot of the six spectral lines shown at the right of Fig. 2 against the data. The widths of the (gaussian) lines are given by $\sigma = 0.158 \mu\text{Hz}$.

unimodal. For each of these eigenvalues there is an eigenvector a_i , containing three components, representing one of the normal modes.

(4) The above three values of $\Delta\omega$ would be observed in a coordinate frame rotating with the solar core. The transformation to an inertial coordinate frame is carried out by again using the rotational operator R . In inertial coordinates (primed) the ingoing wave amplitude (a_i) becomes

$$a'_i = R(\Omega_c t) a_i \quad (9)$$

This splits each normal mode (i) into three components with angular frequencies

$$\omega + \Delta\omega_i + m\Omega_c \quad (10)$$

of different order (m) spherical harmonics, $m = 1, 0, -1$. This is a purely kinematic effect (a rotational Doppler effect). In inertial coordinates the expansion (1) (2) in real harmonics is no longer convenient. Setting

$$\sum a_n W_n = \sum c_m Y_{1,m} \quad (11)$$

the spherical harmonic expansion coefficients c_m are obtained from the matrix equation

$$c = Ta \quad (12)$$

(see Table 1). Here the elements of c are of the order of $m = 1, 0, -1$. As seen from the Earth the (synodic) angular frequency is expression (10) with Ω_c replaced by $\Omega_c - 2\pi/365.25 \text{ (day}^{-1})$.

The expression (10) divided by 2π yields nine different frequencies, three values of $m = 1, 0, -1$ for each normal mode $i = 1, 2$ and 3 . But, as discussed above, the three $m = 0$ terms should be too weak to be observed. Depending on the scattering matrix S and the wave advection angle $\Delta\phi$ in expression (6) the remaining six frequencies can be of different intensities, some being so weak as to be unobserved.

The above discussed origin of the fine structure of the $l=1$ '5-min' normal modes is shown in Fig. 2 using the numerical example discussed below.

The scattering matrix

Evaluated at the surface of the core the intensity of the ingoing wave is proportional to $a^\dagger a$, where $a^\dagger = a^*$ is the Hermitian conjugate of (a). The outgoing wave intensity is $b^\dagger b$ and for a lossless core $b^\dagger b = a^\dagger S^\dagger S a = a^\dagger a$. This implies that $S^\dagger = S^{-1}$, namely that S is unitary.

Because of time reversal invariance in the solar core, for any solution to the wave equation, $\xi(t)$, there is another $\xi^*(-t)$ and for the solution (a, b) there is another (b^*, a^*). Thus, $a^* = S b^*$, $b = (S^*)^{-1} a$, $S = S^*$ and S is symmetric. Owing to these two properties of S , the matrix $i(S+1)(S-1)^{-1}$ is real. Thus its

eigenvectors (and the eigenvectors of S) can be real and S can be diagonalized by an orthogonal matrix.

In the absence of a distortion of the core $S = I$ and for a first-order perturbation by a small core distortion, the correction to S is purely imaginary. This first-order correction to the outgoing wave amplitude (b) is calculated using Green's theorem, neglecting buoyancy effects.

$$\int [\eta \nabla_r \xi_1 - \xi_1 \nabla_r \eta] \rho c^2 \partial_r^2 d\Omega = \int_0^{r_c} \nabla \eta \cdot \nabla \xi_0 C \rho c^2 \partial_r^2 dr d\Omega \quad (13)$$

Here the left side is an integral over the surface of the core, ξ_1 is the first-order perturbation of the radial displacement, $c_0^2(r)$ is the unperturbed sound velocity squared, and $\eta = (f + f^*) W_n \exp(i\omega t)$.

The right side of equation (13) is an integral over the interior of the solar core, ξ_0 is the unperturbed radial displacement and the sound velocity (squared) is $c^2 = c_0^2(r)[1 + C(r, \theta, \phi)]$. The left side yields the perturbation of b_n and consequently the first-order correction to S . Only the $l = 2$ part of C contributes to the right side.

Any real second-degree spherical harmonic ($l = 2$) defines one or more sets of principal axes and can be expressed as a superposition of three second-degree Legendre polynomials, $P_2(n) = \frac{1}{2}(3 \cos^2 \theta_n - 1)$, referred to these three principal axes. The $l = 2$ part of the sound velocity anisotropy $C(r, \theta, \phi)$ can be expressed as

$$C_2(r, \theta, \phi) = h(r) \sum_{n=1}^3 c_n P_2(n) \quad (14)$$

where $\sum_n c_n = 0$.

The right side of equation (13) is easily evaluated with the core first oriented to bring its principal axes into coincidence with the three coordinate axes. Equation (13) then yields a diagonal first-order perturbation of S with elements is_n . The diagonal matrix is designated as S_a , and has the eigenvalues $S_n = \exp(is_n)$ on its diagonal with $s_1 + s_2 + s_3 = 0$.

Substituting equation (14) in (13) and using the WKB approximation gives

$$s_n = -\frac{4\omega c_n}{5} \int_0^{r_c} h(r) \frac{dr}{c_0(r)} \quad (15)$$

To obtain the scattering matrix for the core in its proper orientation, the core is first rotated about the z axis through an angle α , then about the y axis through an angle β and finally again about the z axis. The last rotation can be omitted because it is equivalent to a rotation of the arbitrarily oriented coordi-

nate system about the z axis. The scattering matrix of the reoriented solar core is

$$S = P(\beta) R(\alpha) S_a \tilde{R}(\alpha) \tilde{P}(\beta) \quad (16)$$

(see Table 1 for $P(\beta)$). As noted above, S can be diagonalized by an orthogonal matrix, in this case the matrix (PR) .

A magnetic field in the solar core perturbs the velocity of sound in several different ways, primarily by perturbing the fluid density and pressure, and by the magnetic pressure. For a magnetic field which is primarily toroidal and derived from a spherical harmonic of definite degree (l), all of these contributions to the function $C(r, \theta, \phi)$ have the same angular dependence⁹. This angular dependence can be obtained from the principal moments of inertia of the core which are in turn obtained from the $l = 2$ part of the distortion of the solar surface⁷.

Under a hypothesis to be discussed elsewhere the $l = 2$ part of the surface distortion can be calculated and yields -1.33 ± 0.23 , -0.36 ± 0.22 and 1.69 ± 0.15 ($\times 10^{50}$ g cm²) for the three (traceless) principal moments of inertia of the solar core. (The significance of these results for the nutation and the oscillation of the core is to be discussed elsewhere.) If the above assumptions are valid the three eigenvalue phase angles s_i should be proportional to the above three traceless moments of inertia (I_i) namely

$$s_i/s_j = I_i/I_j \quad (17)$$

and

$$s_i = KI_i$$

Numerical results

Is the fine structure splitting seen in the series of $l = 1$ '5-min' oscillations compatible with a magnetically distorted core rotating with a 12.38-day period (sidereal) as derived from the 1966 observations⁵ of the 'solar oblateness'? Owing to the nutation and a possible long-period oscillation⁹ expected with a magnetically-distorted core, the core orientation obtained from the 1966 data is not expected to be valid in 1981, but the eigenvalue phase angle ratios in expression (17) are assumed to be applicable with a 1σ accuracy. With these restrictions the multiplier K in equation (17) and the angles α , β and $\Delta\phi$ are assumed to be freely adjustable.

By adjusting these four parameters the following conditions should be met: (1) the intensities of the six frequencies with $m = \pm 1$ should divide into three intense spectral lines and three negligible weak ones; (2) the intensity ratios of the strong lines should be acceptable; and (3) their frequency differences should be correct. The example illustrated in Fig. 2 with the parameters given in the caption is considered to be satisfactory. There is no *a priori* reason for the three normal modes to be equally excited and the indicated intensities (assuming a total intensity of 1 for each normal mode) are satisfactory.

In Fig. 3 the six spectral lines shown in Fig. 2 are plotted with the data after adjusting the total intensities of the three normal modes to be in the ratios of 0.985/1.000/0.807 in order of increasing frequency $\Delta\omega_i/2\pi$.

Conclusions

With the assumption that the solar rotation outside the core is substantially uniform, the advection (rotation) angle $\Delta\phi = -0.0166$ permits a calculation of the radius of the core using equation (6). The core is found to have a radius half that of the Sun (3.5×10^{10} cm).

Assuming a distorted mass shell of this radius and the principal moments of inertia given above yields a $\delta\rho/\rho$ contribution to the values of $s_1 \dots s_3$ with magnitudes roughly 1.8 times the above, but with opposite sign. Owing to the important role of the magnetic pressure and the distorted gas pressure, which contribute with signs opposite to $\delta\rho/\rho$, it is impossible to obtain better estimates for s_i without knowing more about the distribution of the magnetic field.

Apparently a magnetically distorted solar core rotating with

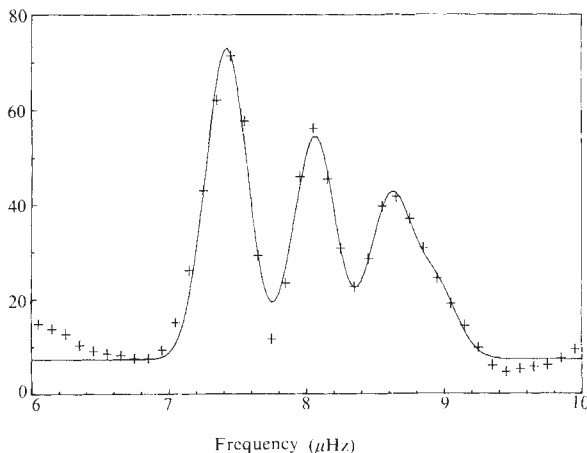


Fig. 4 Same as Fig. 1 but including four Gaussians in the least-square-fit to the data points. In order of increasing frequency the intensity and frequency of each of the three spectral lines are 65.7 ± 2.0 , 7.420 ± 0.006 μHz ; 47.2 ± 2.0 , 8.063 ± 0.008 ; 33.9 ± 2.7 , 8.613 ± 0.018 and the fourth weak peak of unknown cause are 15.1 ± 2.7 , 8.941 ± 0.039 . The difference between the two principal frequency separations is 0.093 ± 0.025 μHz . Thus the two frequency splittings are unequal at a 4σ level.

a 12.38-day period can give a satisfactory account of the triplet structure of the $l = 1$ '5-min' oscillations. But there is one unsatisfactory feature in the above account. The three gaussians fitted to the data in Fig. 1 give equal line splittings for the triplet to within 2σ . But, from the standpoint of the above analysis this is a fortuitous result. We have no explanation for the equality.

But are the splittings between the three spectral lines actually equal? An examination of the high frequency component in Fig. 1 shows a poor fit to the data. There appears to be a blend

with some unidentified weak fourth line, perhaps due to noise or aliasing. By adding a fourth gaussian a satisfactory fit to the high frequency component is obtained (see Fig. 4). Now the splittings of the three principal components are unequal at a statistically significant level (4σ).

For the above interpretation of the $l = 1$ fine structure to be valid, the fine structure would be expected to vary significantly in a year or two, owing to nutation and/or torsional oscillation⁹ of the core.

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Garnet–orthopyroxene barometry for granulites and peridotites

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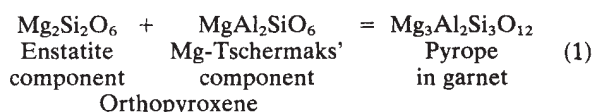
An experimentally calibrated geobarometer based on the solubility of alumina in orthopyroxene coexisting with garnet is presented. This geobarometer is based on experiments in the FeO–MgO–Al₂O₃–SiO₂ and CaO–FeO–MgO–Al₂O₃–SiO₂ systems performed at high temperatures and pressures (800–1,200 °C and 5–20 kbar). Applying the geobarometer to a variety of natural garnet–orthopyroxene assemblages from granulite facies terrains and other occurrences, yields reliable and consistent pressure estimates for these samples.

PETROLOGISTS concerned with garnet peridotite xenoliths in kimberlitic diatremes have long recognized the importance of the solubility of alumina in orthopyroxene coexisting with garnet as a pressure (P)–temperature (T) indicator^{1–4}. More recently, petrologists have applied the garnet–orthopyroxene geobarometer to estimate the physical conditions of equilibration of crustal granulites^{5,6}. Earlier experimental calibrations of the variation in the alumina content of enstatite coexisting with pyrope as a function of P and T in the system MgO–Al₂O₃–SiO₂ (MAS) have been used widely, in conjunction with other geothermometers, to construct 'palaeogeotherms' for mantle-derived garnet peridotites^{7–11}. More recent experimental data in the system MgO–Al₂O₃–SiO₂ (refs 12–16) have tended to confirm the P – T dependence of alumina in enstatite coexisting with pyrope that was obtained by MacGregor⁷. Recent results in CaO–MgO–Al₂O₃–SiO₂ (CMAS)^{13,17} have, however, necessitated a revision in pressures estimated from alumina contents of orthopyroxenes coexisting with pyrope–grossular garnets such as those which occur in garnet peridotites. Major problems encountered in the application of these experimental studies undertaken in simple end member systems to natural rocks, are the effect of the Fe²⁺ contents of the phases on the alumina solubility at any given P – T condition, and the lack of adequate experimental data at P – T conditions directly applicable to natural granulite assemblages. Previous experimental studies in Fe-bearing systems⁵ have been restricted in the compositional range studied, and have yielded only unreversed data for the alumina contents in orthopyroxenes coexisting with a garnet phase. For these reasons, and because of the widespread occurrence of garnet–orthopyroxene assemblages in crustal granulites, we have experimentally recalibrated the garnet–orthopyroxene geobarometer/geothermometer in the FeO–

MgO–Al₂O₃–SiO₂ (FMAS) and CaO–FeO–MgO–Al₂O₃–SiO₂ (CFMAS) systems, over a wide range in X_{Mg} (Mg/(Mg+Fe)) and X_{Ca} (Ca/(Ca+Mg+Fe)) bulk compositions at any one P – T condition, and at P – T conditions appropriate to the formation of crustal granulites (800–1150 °C and 5–20 kbar).

Thermodynamic treatment of garnet–orthopyroxene equilibria

The reaction buffering the alumina content of orthopyroxene coexisting with garnet may be written as^{5,7,14}:



Reaction (1) above is useful for geobarometry because of the large volume change involved ($= 200 \text{ cal kbar}^{-1} \text{ mol}^{-1}$).

The equilibrium condition for reaction (1) at a given pressure, P , and temperature, T , is:

$$\Delta H^\circ - T\Delta S^\circ = RT \ln \frac{(a_{En}^{op})(a_{MgTs}^{op})}{(a_{Py}^{gr})} - P\Delta V^\circ \quad (2)$$

where ΔH° and ΔS° are, respectively, the enthalpy and entropy changes of reaction (1); ΔV° is the standard molar volume change of reaction (1) at 298K; and the a s represent the activities of the components in orthopyroxene and garnet.

The aim of our experimental approach is to extract values of $\Delta H_{1,T}^\circ$ and ΔS_T° from compositional data obtained over a P – T – X (composition) range. To do this we require an expression or value for ΔV° , and a knowledge or model of the activity–composition relationships of the phases involved. While we recognize that full, explicit, thermodynamic models of the garnet and orthopyroxene solid solutions are desirable, and that site distribution data exist for orthopyroxene in some chemical systems^{18,19}, the nature of the data obtainable from

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Table 1 *P-T* estimates for garnet-orthopyroxene assemblages

Locality	Description	Ref.	<i>T</i> (°C)	<i>P</i> (equation (5)) (k bar)	<i>P</i> (other) (kbar)
Greenland	Early Archaean coronites	47	625–700*	9–11.5	> 8.0
Scourie	Metabasites	6	775–835*	6–10.5	silli
Greenland	Late Archaean granulites	48	750, 720*	10, 6.5	7.5
Scourie	Metabasites, coronites	49	725–825*	8, 14.5	silli
Aldan, USSR	Gar granulites	32	925†	10.7–12	silli
Kola, USSR	opx + sill + gar granulites	30	960–1,020†	9–10.5	silli
West Australia	Archaean granulites	50	770†	3.5	silli
Vredefort	Coronas in pseudotachylite	51	700†	5.0	silli
Uganda	Sapphirine bearing granulite	33	1,000†	10	8.1
Nain	Granulites around anorthosite	38	670–859†	2–7	3.3
		39	750 av.	3–4	FaFsQz
Norway	Metadolerites, coronites	52	650*	10–12.5	
Adirondacks	Metadolerites, coronites	53	650*	7.3, 16	8.5, 750 °C
India	Charnockites, mafic granulite	54	800–850‡	7.5–9.5	silli
		55	700–750*	8.4–9.1	silli
		56	850‡	5.0–7.0	7.4
Lesotho	Granulite xenoliths in kimberlite	57	600–700*	11.5–17.5	
Australia	Xenoliths in diatremes	41	900*	14	
		42	925*	12.5	
		43	775*	14	
	Spinel-garnet lherzolites	45	1,200*	22–24	25.1 O'Neill
Arizona	Garnet pyroxenite xenolith	44	710*	15.5	
Enderby Land	Sapphirine-bearing granulites	36	950§	7.0–9.0	7–10
Stanovik, USSR	Aluminous granulites	31	830–875†	4.0–5.2	
			950†	6.5, 9.0	
Ronda, Spain	Recrystallized neoblasts in peridotite massif	58	1,050*	20–21	
			900‡	15–17.5	
Cima de Gagnone	Garnet peridotite body in crustal gneisses	59	900* core	35.0	
			700* rims	22–30	
Selje, Norway	Orthopyroxene eclogite boudin in gneiss	60	650*	18.1	
Lien, Almklovdalen, Norway	Garnet peridotite in ultramafic body in gneiss	61	800* core	32.0	
			750* rims	30.1	
		62	650*	20.2	
Scourie	Metagneous granulites	63	840*	9.3	
			875*	11.0	840 °C, 10.7
			860*	8.2	gar-bi
			800*	7.0	gar-opx-
			780*	6.4	plag-qz
			745*	6.7	
			700*	5.0	

* Garnet-clinopyroxene thermometer⁶⁴. † Garnet-orthopyroxene thermometer⁶⁵. ‡ Two-pyroxene thermometer³⁴. § Phase petrology constraints.

the experiments presented here precludes the use of complicated multi-parameter solution models. A simpler approach which allows direct application to natural rock data has been pursued. Simple solution models applicable in the MAS and CMAS systems have been adopted, and essentially empirical curve-fitting parameters which describe the compositional dependences of Al-solubility in orthopyroxene in the Fe-bearing systems have been added. By taking the solid solution properties of the phases into account, the empirical terms have a similar form to terms describing non-ideal solid solution behaviour in more rigorous formulations.

In the MAS system, the activity of garnet is unity. Using the two-site model of Wood⁵ for orthopyroxene, and assuming that no Al enters the M2 site:

$$a_{\text{En}}^{\text{op}} a_{\text{MgTs}}^{\text{op}} = X_{\text{Al}}^{\text{M1}} (1 - X_{\text{Al}}^{\text{M1}}) \gamma_{\text{Mg}}^{\text{M1}} \gamma_{\text{Al}}^{\text{M1}}$$

Recent work indicates that some Al may enter the M2 site¹⁹, however, the simpler model above is adopted to allow development of a practical geobarometer.

Following Wood and Banno²⁰, Mg-Al orthopyroxenes have been assumed to mix ideally at 1 bar, and non-ideal interactions at higher pressure have been arbitrarily ascribed to an excess volume of mixing term ΔV_{ex} . Substitution of this term, derived from available molar volume data^{14,21}, for the standard molar volume change of reaction (ΔV°), results in a *P-T*-dependent volume change of reaction, ΔV_r :

$$\Delta V_r = -[183.3 + 178.98(X_{\text{Al}}^{\text{M1}}(1 - X_{\text{Al}}^{\text{M1}}))] \text{ cal kbar}^{-1} \quad (3)$$

Thus, in MAS:

$$\Delta G_{1,T}^\circ = RT \ln [X_{\text{Al}}^{\text{M1}}(1 - X_{\text{Al}}^{\text{M1}})] - P \Delta V_r$$

With the addition of Fe into the system, both garnet and orthopyroxene vary in composition with *P* and *T*. In orthopyroxene several effects will arise from non-ideal interactions occurring on either 'M1' or 'M2' site or across sites (reciprocal terms). While recognizing these complexities and that garnet is no longer a pure component, the effects of adding Fe to the MAS system have been ascribed to one, empirical, curve-fitting term which accounts for the variation of Al-solubility in orthopyroxene with $X_{\text{Fe}}^{\text{op}}$ at one *P-T* condition (Fig. 1). This excess term is dominated by non-ideal Fe-Al interactions in the M1 site of orthopyroxene and is of the form:

$$\Delta H_{\text{ex}} = W_{\text{FeAl}}^{\text{M1}} (1 - X_{\text{Al}}^{\text{M1}}) (1 - 2X_{\text{Al}}^{\text{M1}}) X_{\text{Fe}}^{\text{op}}$$

with

$$X_{\text{Fe}}^{\text{op}} = \text{Fe/Mg} + \text{Fe in orthopyroxene and}$$

$$X_{\text{Al}}^{\text{M1}} = \text{Al}/2 \text{ in 6 oxygen unit orthopyroxene}$$

Note that the $W_{\text{FeAl}}^{\text{M1}}$ parameter derived here is an empirical term only, and its magnitude is the net result of a number of effects in the orthopyroxene solid solution. Nevertheless, the ΔH_{ex} term adequately describes the deviation of Al-contents of Fe-Mg orthopyroxenes coexisting with garnet from those of an Mg-orthopyroxene at the same *P-T* condition.

In the CFMAS system, further terms must be introduced to account for the decrease in alumina contents of orthopyroxenes

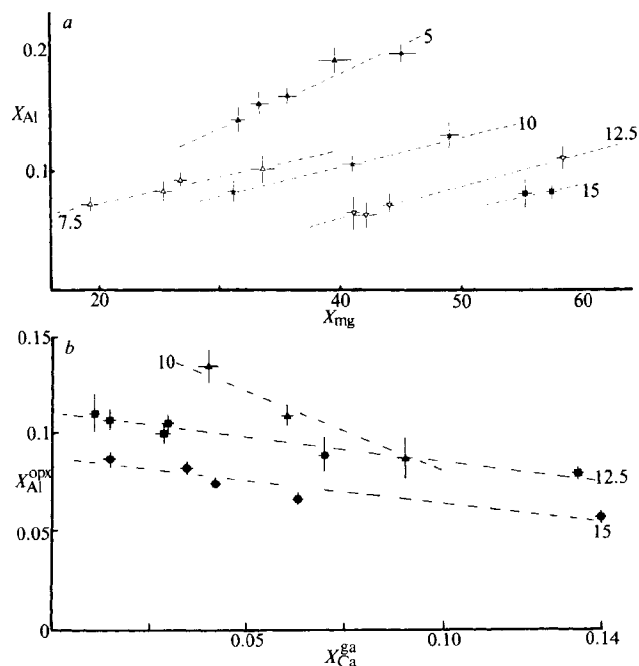


Fig. 1 *a*, Example of an experimental data set in the FMAS system showing the dependence of Al ($X_{Al} = \text{Al}/2$) solubility in orthopyroxene on Fe–Mg substitution in the pyroxene at 1,248K. Dashed lines join isobaric data, with pressures as indicated. Similar data has been obtained at 1,173K, 1,323K and 1,423K. $X_{Mg} = (\text{Mg}/\text{Mg} + \text{Fe}) \times 100$ in orthopyroxene. Error bars, one standard deviation (1σ) based on 10–15 orthopyroxene analyses. *b*, Example of an experimental data set in the CFMAS system showing the dependence of Al-solubility in orthopyroxene on the grossular content of coexisting garnet, at near-constant $X_{Mg}^{\text{opx}} (X_{Mg}^{\text{opx}} = 60)$ and at 1,248K. Dashed lines join isobaric data, with pressures as indicated. Similar data has been obtained at 1,323K. $X_{Ca}^{\text{ga}} = (\text{Ca}/\text{Ca} + \text{Mg} + \text{Fe})$ in garnet.

with increasing Ca-contents of coexisting garnet. In comparison with the FMAS equation used above, where X_{py}^{ga} is left as unity, in CFMAS the equation is adjusted using two terms:

- (1) A dilution term incorporated into the expression for $\ln K$

$$X_{py}^{\text{ga}} = (1 - X_{Ca}^{\text{ga}})^3$$

- (2) An additional term derived from a ternary regular-symmetric solid solution model for Ca–Mg–Fe garnets, with the constraints obtained from other phase equilibrium experiments that Ca–Fe and Fe–Mg interactions in garnet are near-ideal in the P – T range of interest^{22–24}:

$$RT \ln \gamma_{py}^{\text{ga}} = W_{CaMg}^{\text{ga}} [X_{Ca}^{\text{ga}} X_{Fe}^{\text{ga}} + (X_{Ca}^{\text{ga}})^2]$$

where

$$X_{Ca}^{\text{ga}} = \text{Ca}/\text{Ca} + \text{Mg} + \text{Fe}, \text{ and } X_{Fe}^{\text{ga}} = \text{Fe}/\text{Ca} + \text{Mg} + \text{Fe}.$$

Note that this second term results in a derived W_{CaMg}^{ga} which is a curve-fitting parameter only, as the term $(1 - X_{Ca}^{\text{ga}})$ rather than X_{Mg}^{ga} has been used for the mole fraction of garnet. Even though the derived W_{CaMg}^{ga} is an empirical parameter, the equations used here should give the correct form of Ca–Mg interaction in garnet.

Thus, in CFMAS an equation is derived which expresses the alumina content of orthopyroxene coexisting with garnet as a function of P , T , X_{Fe}^{op} , X_{Ca}^{ga} and X_{Fe}^{ga} :

$$\begin{aligned} \Delta G_{1,T}^{\circ} = & RT \ln [X_{Al}^{\text{M1}} (1 - X_{Al}^{\text{M1}}) / (1 - X_{Ca}^{\text{ga}})^3] - P \Delta V_r \\ & + W_{FeAl}^{\text{M1}} (1 - X_{Al}^{\text{M1}}) (1 - 2X_{Al}^{\text{M1}}) X_{Fe}^{\text{op}} \\ & + 3W_{CaMg}^{\text{ga}} [X_{Ca}^{\text{ga}} X_{Fe}^{\text{ga}} + (X_{Ca}^{\text{ga}})^2] \end{aligned} \quad (4)$$

The development of this equation has essentially relied on the application of compositional correction factors, derived from

either the orthopyroxene (in FMAS) or the garnet (in CFMAS) solid solutions, to a simple equilibrium relation applicable in MAS, and thus the derived parameters are partly empirical and may incorporate a greater number of more subtle effects in the solid solutions considered.

Experimental data on garnet–orthopyroxene equilibria

Equation (4) may be used in the reduction of experimental data obtained over a range of P – T – X_{Mg}^{bulk} – X_{Ca}^{bulk} conditions, given that values of X_{Al}^{M1} , X_{Mg}^{op} , X_{Ca}^{ga} and X_{Fe}^{ga} can be obtained experimentally. In this experimental study, two sets of experiments designed to obtain data sets for these compositional parameters have been undertaken:

(1) Experiments in the system FMAS at a range of X_{Mg}^{bulk} values ($= 0.15, 0.30, 0.45, 0.50, 0.60, 0.70, 1.0$), with bulk compositions appropriate to form garnet–orthopyroxene–quartz at a given P – T condition. These have been performed with both almandine and Al-free enstatite mineral mixes ($X_{Mg}^{\text{bulk}} = 0.33$), and glass mixes seeded with 5% almandine or pyrope to promote garnet growth. It is considered that mineral-mix type starting materials constitute reversal mixes to glass starting materials, an assumption confirmed by analyses of orthopyroxenes grown from glass (X_{Al}^{M1} decreases rimwards) and from enstatite seeds (X_{Al}^{M1} increases rimwards).

(2) Experiments in CFMAS at $X_{Mg}^{\text{bulk}} = 0.50$ and over a range of X_{Ca}^{bulk} values ($= 0.02, 0.04, 0.08$) so as to produce garnets of differing X_{Ca}^{ga} at essentially one P – T – X_{Mg}^{op} condition. In these experiments, glass starting materials with 5% almandine seeds have been used exclusively, and the effect of X_{Ca}^{ga} on X_{Al}^{M1} has been evaluated by comparison of compositional data in this set of experiments with data previously obtained and reversed in FMAS.

All experiments have been performed in a Boyd-type, 1/2-inch, solid-medium, piston–cylinder apparatus. Temperatures were monitored by Pt/Pt90Rh10 thermocouples to within $\pm 10^\circ\text{C}$ of the set point during the prolonged run durations involved in ensuring subsolidus equilibration (4 days to 1 month, depending on the temperature). Pressures, monitored by Heise gauge, are believed to be accurate to within 0.5 kbar. All runs were of piston-in type, and a -10% friction correction has been applied to all nominal gauge pressures as talc outer sleeves were used. All samples were loaded into 3- or 4-bore Fe-metal or graphite capsules which were then sealed in noble metal outer capsules. Thus three or four mixes of differing bulk composition could be simultaneously run at one P – T condition.

Examples of the effects of X_{Mg}^{op} and X_{Ca}^{ga} on X_{Al}^{M1} , at various pressures for a given temperature, are illustrated in Fig. 1. The

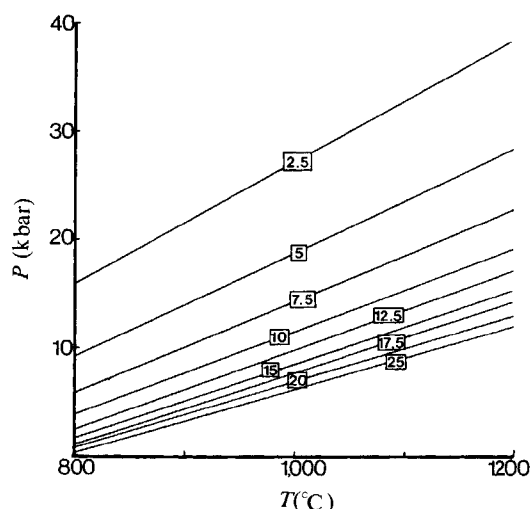


Fig. 2 X_{Al} (Al/2) isopleths for hypothetical orthopyroxene with $X_{Mg}^{\text{op}} = 50$, in equilibrium with Fe–Mg garnet. Isopleths are expressed as % Al in the M1 site.

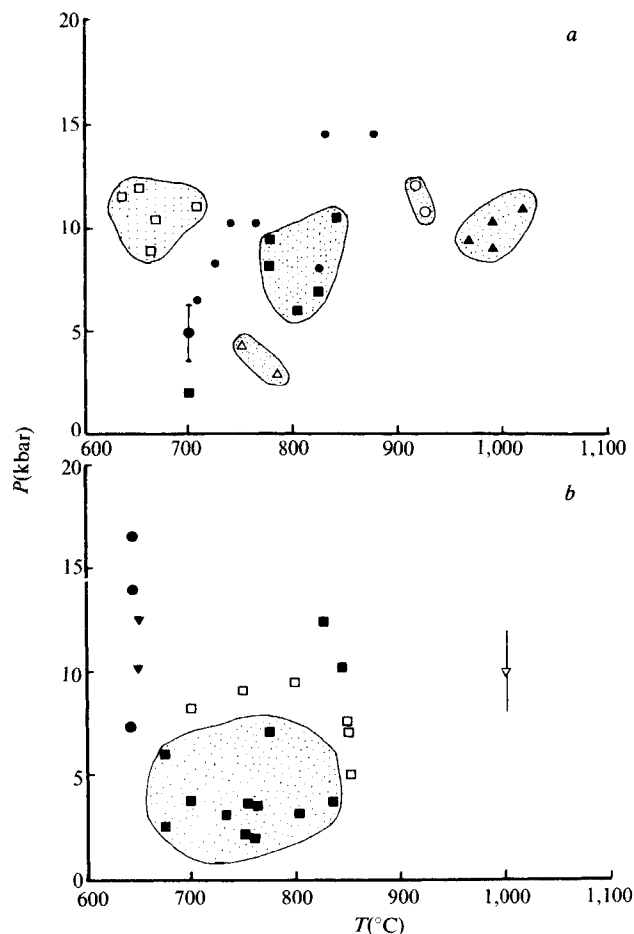


Fig. 3 P - T estimates for published examples of garnet-orthopyroxene-bearing granulites. P estimated from equation (5). T estimates as indicated in Table 1. *a*: $\square$, Greenland⁴⁷; $\blacksquare$, Scourie⁶; $\circ$, Aldan Shield³²; $\blacktriangle$, Kola Peninsula³⁰; $\triangle$, West Australia⁵⁰; $\bullet$, Vredefort⁵¹; $\blacklozenge$, Greenland and Scotland^{48,49}. *b*, $\blacksquare$, Nain, Labrador^{38,39}; $\blacklozenge$, Norway⁵²; $\bullet$, Adirondacks⁵³; $\square$, India⁵⁴⁻⁵⁶; ∇ , Uganda³³.

principal effects to be noted are:

$X_{\text{Al}}^{\text{M1}}$ decreases with increasing P at constant T , $X_{\text{Mg}}^{\text{op}}$ and $X_{\text{Ca}}^{\text{ga}}$

$X_{\text{Al}}^{\text{M1}}$ increases with increasing T at constant P , $X_{\text{Mg}}^{\text{op}}$ and $X_{\text{Ca}}^{\text{ga}}$

$X_{\text{Al}}^{\text{M1}}$ increases with increasing $X_{\text{Mg}}^{\text{op}}$ at constant P , T and $X_{\text{Ca}}^{\text{ga}}$

$X_{\text{Al}}^{\text{M1}}$ decreases with increasing $X_{\text{Ca}}^{\text{ga}}$ at constant P , T and $X_{\text{Mg}}^{\text{op}}$

The experimental data in FMAS, a total of 75 data points, have been fitted to equation (4), with $X_{\text{Ca}}^{\text{ga}} = 0.0$, yielding best-fit multiple and stepwise regression parameters as follows:

$$\Delta H_{1,T}^{\circ} = -5,650 \pm (250) \text{ cal mol}^{-1}$$

$$\Delta S_T^{\circ} = -2.93 \pm (0.30) \text{ cal mol}^{-1} \text{ K}^{-1}$$

$$W_{\text{FeAl}}^{\text{M1}} = 5,157 \pm (200) \text{ cal mol}^{-1}$$

(figures in brackets are standard errors at the 1σ level).

Compositional data in CFMAS at $X_{\text{Mg}}^{\text{op}} = 0.60$ have then been fitted to equation (4) using the parameter values above, to obtain a least-square fit value of $W_{\text{CaMg}}^{\text{ga}}$

$$W_{\text{CaMg}}^{\text{ga}} = 2,100 \pm (200) \text{ cal mol}^{-1}$$

Correction of equation (4) for excess molar volume terms in CaMg garnet²⁵, leads to a value of $2,530 \pm 200 \text{ cal mol}^{-1}$ for this empirical parameter, in reasonable accord with values of $W_{\text{CaMg}}^{\text{ga}}$ obtained in other studies^{24,26,27}.

Equation (4) may now be rearranged into an equation expressing P (kbar) in terms of T , $X_{\text{Mg}}^{\text{op}}$, $X_{\text{Al}}^{\text{M1}}$, $X_{\text{Ca}}^{\text{ga}}$ and $X_{\text{Fe}}^{\text{ga}}$.

This equation is useful as a geobarometer in granulite facies garnet-orthopyroxene assemblages and in garnet periodotites:

$$P \text{ (kbar)} = \frac{1}{\Delta V_r} [(R \ln K - 2.93)T + 5,650 + 5,157(1 - X_{\text{Al}}^{\text{M1}})(1 - 2X_{\text{Al}}^{\text{M1}})X_{\text{Fe}}^{\text{op}} - 6,300[X_{\text{Ca}}^{\text{ga}}X_{\text{Fe}}^{\text{ga}} + (X_{\text{Ca}}^{\text{ga}})^2] \quad (5)$$

where T is in (Kelvin; $K = [X_{\text{Al}}^{\text{M1}}(1 - X_{\text{Al}}^{\text{M1}})/(1 - X_{\text{Ca}}^{\text{ga}})^3]$; $\Delta V_r = -[183.3 + 178.98(X_{\text{Al}}^{\text{M1}}(1 - X_{\text{Al}}^{\text{M1}}))] \text{ cal kbar}^{-1}$.

The geobarometer will be limited by the following circumstances: (1) where Fe^{3+} contents are high in garnet or orthopyroxene, such that other non-ideal terms may be important in the garnet, or the $\text{Fe}^{3+}\text{AlMg}_{-1}\text{Si}_{-1}$ exchange may be important in orthopyroxene^{28,29}; (2) where Cr^{3+} contents are high in garnet; (3) where Mn^{2+} contents are high in the phases.

In addition, the barometer will be subject to considerable uncertainty when either (1) Al_2O_3 contents in orthopyroxene are very low (such as high pressure garnet peridotites, or high Fe-Ca garnet assemblages formed at low temperatures), or (2) there are large uncertainties in the temperature estimate, as the dP/dT slopes of the Al_2O_3 isopleths are approximately 4 kbar per 100 °C (Fig. 2).

Applications of garnet-orthopyroxene geobarometry

The garnet-orthopyroxene geobarometer developed here (equation (5)) has been applied to several well-documented examples of granulite facies metamorphics, and to low-Cr garnet peridotite xenoliths in kimberlites from South Africa. The results of these calculations are presented in Fig. 3 and Table 1, together with the P - T conditions suggested by other workers for these samples on the basis of other geothermobarometric techniques or phase stability constraints.

Sillimanite- or sapphirine-bearing garnet-orthopyroxene assemblages reported from the USSR³⁰⁻³² and Uganda³³ yield P estimates which are consistent with the stability of sillimanite and with the stability of the assemblage orthopyroxene-sillimanite-quartz at 1,000 °C (ref. 34). Granulites from Enderby Land, Antarctica³⁵, yield pressures of 7-9 kbar, consistent with the stability of sillimanite in interlayered metapelites and with phase assemblages in associated aluminous granulites³⁶.

Retrograde garnet-bearing coronas in meta-igneous granulites from several areas (Table 1) yield pressure estimates in the range 7-12 kbar, which is consistent with the reported incoming of kyanite in metapelites associated with these coronas in some areas³⁷. Unrealistic or highly uncertain P estimates are obtained, however, for some of these corona textures (for example, Adirondacks, Scourie). This may result from inadequacies in the experimental calibration at extreme Ca- and Fe-rich compositions, from the large errors inherent in the barometry at low $X_{\text{Al}}^{\text{M1}}$ values, or from failure to obtain adjacent analyses of minerals in local equilibrium in otherwise zoned coronas. The Nain³⁸, Labrador, granulites, which occur in a thermal aureole around an anorthosite pluton, yield an average pressure estimate consistent with pressures of 3.2 kbar recently obtained for these rocks from olivine-orthopyroxene-quartz equilibria^{39,40}.

Granulitic and websteritic nodules from diatremes in southeastern Australia and the USA⁴¹⁻⁴⁴ yield pressures of 12.5-16 kbar, which are in good agreement with pressures inferred by Irving⁴² for the equilibration of scapolite granulites from Delegate, New South Wales. Spinel-garnet lherzolite xenoliths from southeastern Australia⁴⁵, estimated to have equilibrated at 25 kbar by O'Neill⁴⁶ using the spinel to garnet peridotite transition as a barometer, yield pressures of 22-24 kbar using equation (5).

Comparison with most alternative pressure estimates considered here indicates that the experimentally derived

geobarometer presented in equation (5) can be used successfully to estimate the pressures of formation and equilibration of garnet-orthopyroxene assemblages, provided a reliable temperature estimate can be obtained from other equilibria. Calculated pressures will, however, be heavily dependent on the deduced temperature. As in all geothermometry/geobarometry using several equilibria, it is also necessary to assume that

closure temperatures are the same for the equilibria used.

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Relaxation of muscle fibres by photolysis of caged ATP

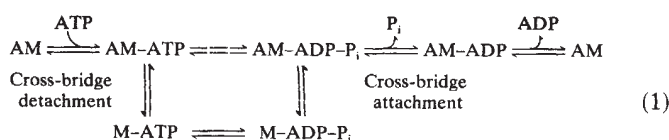
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A novel method has been developed for studying the reaction kinetics of the force-generating mechanism in muscle. Inert photolabile precursors of ATP or ADP are incorporated into muscle fibres having their surface membrane barrier removed. The nucleotide is then rapidly liberated by laser pulse photolysis. This circumvents the limitation in time resolution set by diffusion of nucleotide from the medium bathing the fibre. This laser photolysis method may be applicable to studies of the dynamic properties of many biological systems.

THE energy transducing mechanism of muscle is widely thought to involve a cyclic reaction of cross-bridges between thick and thin filaments^{1,2}. Lymn and Taylor proposed a scheme relating the chemistry of the actomyosin ATPase to muscle contraction³. This scheme, expanded to include some recently characterized steps is⁴⁻⁶:



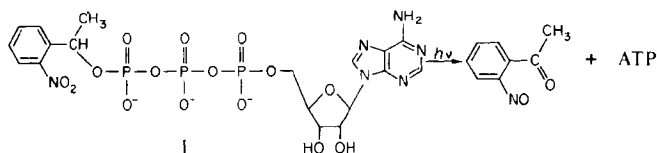
Kinetic studies using the isolated proteins indicate that ATP

binds to the AM state (actomyosin with no bound nucleotide) and induces rapid detachment of the myosin head from actin⁷. We have used a laser photolysis method to study the corresponding reaction in muscle fibres capable of contracting. Muscle fibre tension and high frequency stiffness were monitored after rapid increases in ATP concentration to determine the kinetics of the mechanical events that follow binding of nucleotide to the cross-bridges.

Although solution experiments have demonstrated the reversibility of the actin-myosin association and dissociation reactions, and direct ATP hydrolysis without dissociation (shown above as dashed arrows)^{8,9}, for clarity of the experimental description, treatment of these phenomena is deferred to the discussion.

Photochemical initiation of cross-bridge detachment

The tension record of Fig. 1 illustrates the experimental procedure. A skinned single fibre from rabbit skeletal muscle was initially relaxed (solution 1). Transfer to a medium without ATP (solution 2) resulted in tension development as cross-bridges accumulated in the rigor (AM) state. The inert precursor of ATP, P^3 -1-(2-nitro)phenylethyladenosine 5'-triphosphate, **1**, termed caged ATP¹⁰, was added to the bathing medium (solution 3) and allowed to diffuse into the fibre. This caused little alteration of the rigor tension. At the arrowhead of Fig. 1 a pulse of UV radiation ($\lambda = 347$ nm) from a frequency-doubled ruby laser initiated the photolysis reaction¹¹:



In the experiment shown in Fig. 1, 750 μ M ATP was liberated in the muscle chamber. The rate of ATP release, set by photochemical dark reactions¹¹, was 100 s^{-1} in the solutions used in this study. Production of ATP in the presence of excess Mg^{2+} ions but no added Ca^{2+} resulted in a rapid decrease of tension to the relaxed level. Figure 1*b* shows the same tension decay on a faster time base. The arrowhead indicates the laser pulse. After a brief delay, partly due to the photochemical reaction, tension decreased with a half time ($t_{1/2}$) of ~ 5 ms. Thus ATP detaches cross-bridges rapidly in muscle fibres.

To make a kinetic comparison of cross-bridge detachment with the rate of actomyosin dissociation in solution, the amount of ATP liberated was varied by altering the intensity of the light pulse. Figure 2*a-c* shows transients of relaxation from rigor for several ATP concentrations. The upper traces in each panel are records of the high-frequency mechanical stiffness of the fibres, which is a qualitative indication of the number of cross-bridge attachments¹². After the light pulses (arrows), the stiffness decayed, indicating cross-bridge detachment. Accompanying the stiffness decrease there was a delayed rise in force and then a decrease to the relaxed level (lower traces). As the ATP concentration was reduced, the peak in tension was delayed, the amplitude of the tension rise increased and the final relaxation was slower.

The time taken for the force to decay from the peak of the tension rise to half that value was converted to an apparent rate constant using the relation $k_{obs} = (\ln 2)/t_{1/2}$. This method of analysis ignores deviations of the final tension decay from a single exponential but is useful for comparison with the analogous experiment on actomyosin in solution. The rate of the final relaxation increased as the ATP concentration increased (see Fig. 2*d*). The slope of this relation, K , is a second-order rate constant for the overall detachment process of rigor cross-bridges induced by ATP. The average value for five fibres was $K = 1.9 \pm 0.6 \times 10^5 M^{-1} s^{-1}$ (mean $\pm$ s.d.) at 20 °C.

When acto-heavy meromyosin or actomyosin subfragment-1 is mixed in solution with ATP in a rapid reaction apparatus, and the dissociation of the myosin heads is monitored by decrease in light scattering, the apparent second-order rate constant for dissociation is $\sim 10^6 M^{-1} s^{-1}$ (ref. 13, using acto-heavy meromyosin at ionic strength = 100 mM, $T = 20$ °C). Thus, our data indicate that in a fibre the rate of cross-bridge detachment from the rigor state is within an order of magnitude of the rate of the corresponding reaction with solubilized proteins.

The sensitivity of the detachment reaction to stress was tested by varying the length of the muscle fibre in rigor before photolysis. Figure 3 shows superimposed tracings of records obtained

when small length steps were applied 1 second before the laser pulse. This altered the distribution of forces in the attached cross-bridges, which is apparent in the alteration of the shape of the initial 20 ms of the force transient. For Fig. 3*a*, the muscle fibre was stretched ~ 30 μ m (0.86% of the initial length) before the laser pulse. Photolysis resulted in an initial rapid decrease in tension. The force imposed by the stretch was similar to the force reached during an isometric contraction of these glycerinated muscle fibres. No length change was applied for Fig. 3*b*. Figure 3*c, d* was recorded after the length was allowed to decrease. After the laser pulse, tension remained constant or increased before the final relaxation. Although the initial phase of these transients varies greatly in shape, the final phases superimpose.

Initial increase in tension

An initial tension rise was generally apparent if the tension was low (< 50 mN mm^{-2}) at the time of the laser pulse. This low rigor tension level could be achieved either by decreasing the length of the muscle fibre just before the laser was triggered (for example, Fig. 3*d*) or by initially inducing rigor in a medium free of divalent metal ions and containing EDTA¹⁴. The initial rise in tension after liberation of ATP inside the myofibrils was

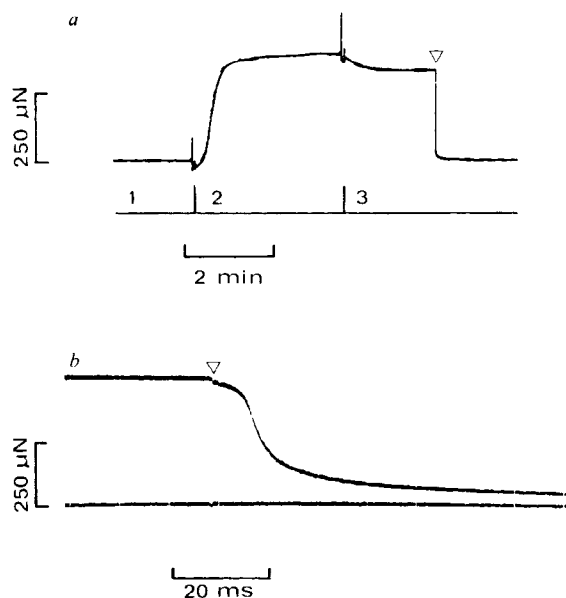
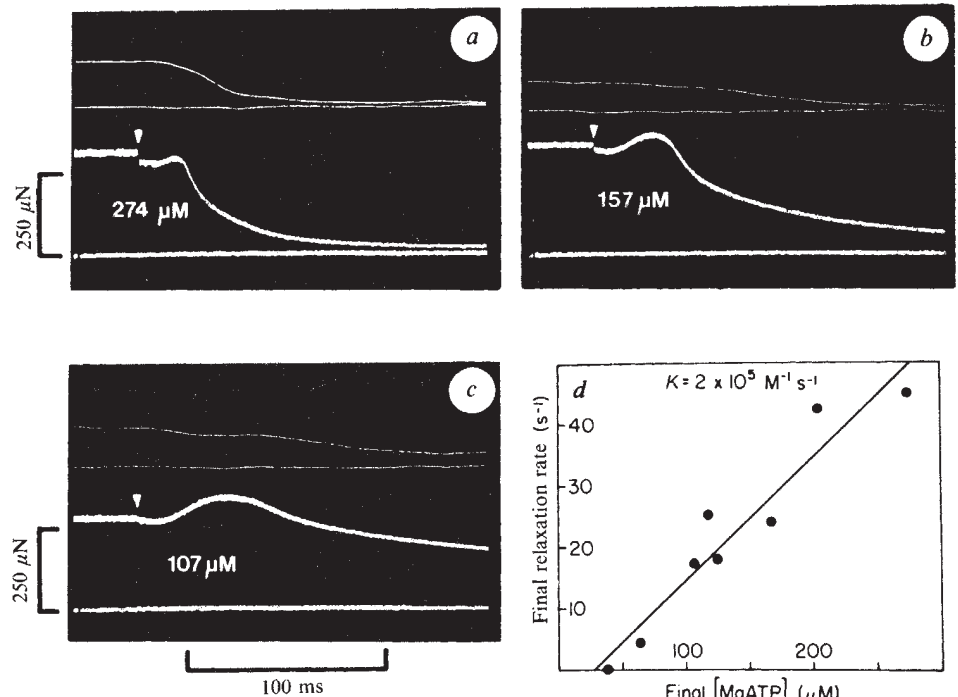


Fig. 1 Tension transient recorded from a single glycerol-extracted rabbit psoas muscle fibre. *a*, The slow time base tension record illustrates the experimental protocol. The fibre (cross-sectional area $7.95 \times 10^{-9} m^2$, sarcomere length $2.3 \mu m$) was attached with aluminium clips between a force transducer (natural frequency, 7.5 kHz) and a piezoelectric stack which was used to alter muscle length. The fibre was immersed in a small trough, ~ 0.5 mm from a quartz window. Bars on the record indicate the timing of the solution exchanges. (1) Relaxing solution containing (in mM): MgATP, 0.1; Mg^{2+} , 1; K^+ , 95; EGTA, 30; creatine phosphate (CP), 22; creatine kinase (CK), 1 mg ml^{-1} ; TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid) buffer, 100; reduced glutathione, 10; ionic strength, 200; pH 7.1; 21 °C). (2) Rigor solution—same as (1) but without ATP, CP or CK. (3) Photolysis solution—same as (1), but without ATP and including 10 mM caged ATP. Photolysis of caged ATP was initiated at the arrowhead by a 150-mJ, 50-ns pulse of 347 nm light generated by frequency-doubling the output of a 694 nm ruby laser with a temperature-tuned rubidium dihydrogen arsenate crystal. After the rapid relaxation induced by the release of ATP, the photolysis solution was saved and later assayed for ATP using luciferin-luciferase^{35,36}. *b*, Fast time base recording of the tension transient illustrated in *a*. The arrowhead indicates the time of the laser pulse. The lower trace is a baseline tension recorded after relaxation in the photolysis solution.

Fig. 2 Time course of relaxation at various concentrations of ATP. *a-c*, Paired stiffness (upper) and tension (lower) records. Lower traces of each pair show the baseline level in the relaxing solution. The degree of photolysis of the initial 2 mM caged ATP was controlled by varying the intensity of the light pulse¹¹. Final ATP concentrations are shown for each panel. Stiffness was recorded by altering fibre length sinusoidally at 2.5 kHz and 1 μm amplitude with the piezoelectric stack. The sinusoidal component of the tension recorded in phase with the length change was separated from the d.c. component of tension by a synchronous demodulator. Nonlinear end compliance and sinusoidal phase shift due to stress relaxation may introduce small artefacts into the stiffness records. *d*, Observed rate of final relaxation of tension determined from the $t_{1/2}$ as described in the text. The data are from a single fibre. The line was fitted by linear regression, slope $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The non-zero intercept of the line is probably a reflection of the relatively high concentration of myosin heads within the fibre.



unexpected as mixing MgATP with actomyosin in solution causes rapid dissociation of the myosin head from actin filaments^{5,7,13}.

Figure 4a shows a control experiment with caged ADP that was performed to determine whether the shape of the photolysis-induced mechanical transient was caused by an artefact of the method. The photochemical species and accompanying reactions were identical with those in a caged ATP experiment except that 240 μM ADP was released. Little alteration in tension or stiffness occurred. However, when the liberated ADP was enzymatically converted to ATP by including creatine phosphate (CP) and creatine kinase (CK) in the medium, a tension rise and relaxation were observed (Fig. 4b). This indicates that these phenomena were caused solely by the appearance of ATP in the filament lattice.

A possible explanation of the initial increase in tension before relaxation that cross-bridges transiently reattach to the thin filaments shortly after detachment. If the reattached links produce more tension than the detaching cross-bridges, then tension increases. The stiffness (upper traces in Fig. 2a-c and Fig. 4b) declines during the initial tension rise, which indicates that the combination of detachment and reattachment results in a net detachment throughout the transient.

A rapid reattachment of detached cross-bridges might be allowed even in the absence of Ca^{2+} ions by a cooperative effect among thin filament attachment sites which has been demonstrated in other studies¹⁵⁻¹⁷. The dependence of the ATPase activity of rabbit myosin and regulated thin filaments (actin combined with troponin and tropomyosin) on ATP concentration and the relationship between the steady tension of skinned muscle fibres and ATP concentration are both bell-shaped with maxima at substrate concentrations of 2-30 μM ¹⁵⁻¹⁹. These data can be explained on the basis of a cooperative interaction between thin filament attachment sites such that, if a proportion of cross-bridges are attached in rigor (nucleotide-free) bonds, then the remainder of sites are available for cross-bridge attachment irrespective of Ca^{2+} binding to troponin^{15,16}. This cooperativity is consistent with the structure of the thin filaments, since tropomyosin regulates several actin monomers²⁰. Interactions between the two heads of the myosin molecule might also account for cooperative attachment.

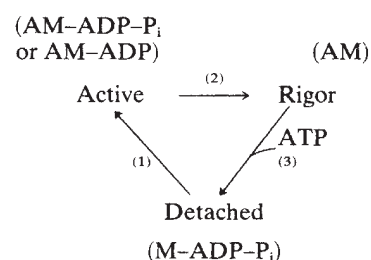
If cooperativity is to account for the tension rise, cross-bridges must be capable of detaching, reattaching and producing force

within the 20-30-ms period between the illumination and the peak of the tension rise (Figs 2a-c and 3). The rate of reattachment and force generation can be tested by including Ca^{2+} ions in the photolysis medium, as liberation of ATP in the presence of Ca^{2+} will activate the muscle fibre to contract. If reattachment of cross-bridges occurs slowly compared with the detachment in this condition, then the muscle fibre should relax fully before the active high tension state is reached.

In Fig. 5 the tension record labelled $-\text{Ca}^{2+}$ shows a relaxation from rigor initiated by photolysis of caged ATP in the absence of Ca^{2+} . The length of the muscle fibre was allowed to decrease by 1% 1 s before the photolysis and a moderate concentration (190 μM) of ATP was generated in the fibre so that a significant tension rise would occur. In the next trial the fibre in rigor was bathed in a solution containing caged ATP and $\sim 30 \mu\text{M}$ free Ca^{2+} . Addition of Ca^{2+} ions had little effect on the rigor tension or stiffness. When ATP was released in the presence of Ca^{2+} , the tension rose towards the level of an active contraction. The two force traces diverge within 20 ms after the laser pulse, indicating that the cross-bridges can generate additional force above the rigor level within 20 ms of ATP release. Assuming that rigor cross-bridges detach during this period, the results suggest that reattachment and force generation also occur rapidly.

Kinetic model

The possibility that a rapid reattachment of detached cross-bridges could account for the shape of the force transients was tested by using a restrictive model involving a minimum number of steps. Three cross-bridge states were considered in this calculation: detached, rigor and active. These are shown in the following scheme together with the probable corresponding chemical intermediates:



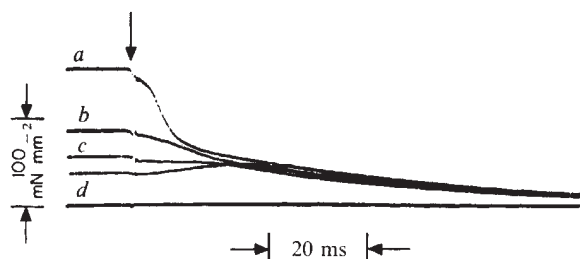


Fig. 3 Superimposed records of relaxation transients produced by photoliberation of $\sim 480 \mu\text{M}$ ATP. The muscle length (3.5 mm; sarcomere length $2.32 \mu\text{m}$) was changed slightly 1 s before the laser pulse. Length steps for each trace were: a, $30 \mu\text{m}$ stretch; b, no length step; c, $15 \mu\text{m}$ release; d, $30 \mu\text{m}$ release. Other conditions were as described in Fig. 1 legend. Units of tension per cross-sectional area are given for the force calibration to facilitate comparison with active contractions of glycerinated muscle fibres. The vertical positions of the records were adjusted to superimpose the four baselines (lower trace) showing the relaxed tension.

To introduce cooperativity into the model, the cross-bridge attachment sites were considered in pairs. For each member of a pair, reaction (1), the cross-bridge attachment step, was made to depend on the state of the other attachment. If one site of a pair was attached, then the attachment reaction at the other site was allowed (see Fig. 6 legend). For simplicity, the rate constants of these reactions were made independent of cross-bridge stress and strain. The resulting differential equations were integrated. The attached cross-bridge states were assigned values of force, given in Fig. 6 legend. The concentrations of the states, weighted by these relative forces, were summed to produce simulated curves of the tension time courses.

To simulate the experiments in which a stretch or release was applied to the muscle before the photolysis pulse (Fig. 3), the rigor cross-bridges present at the time of photolysis were given different values of force for each curve in Fig. 6. The

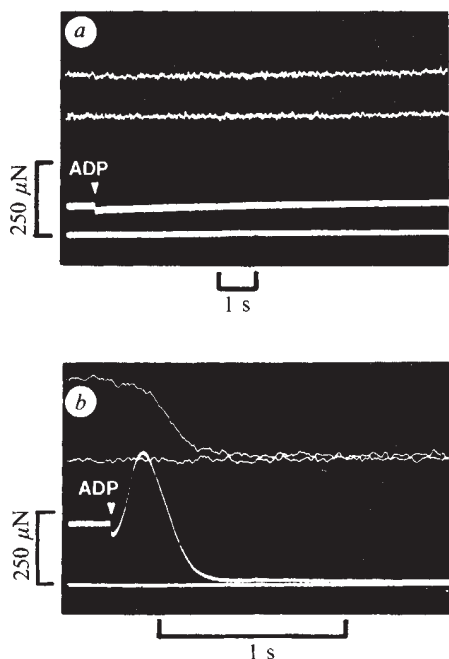


Fig. 4 Tension (lower) and stiffness (upper) records during photolysis of caged ADP. a, Caged ADP in the absence of CP and CK. Final ADP concentration was $240 \mu\text{M}$. b, Photolysis of caged ADP in the presence of CP (22 mM) and CK (1 mg ml^{-1}). Final ATP concentration, $290 \mu\text{M}$. Conditions and baseline recordings (lower tension and stiffness traces) were as in Fig. 1 except that caged ATP was replaced by 2 mM caged ADP.

shape of the initial portion of the curves depended strongly on the force per rigor cross-bridge. When the initial tension was low, detachment of rigor cross-bridges had a relatively small effect on the calculated tension curve and reattaching cross-bridges generated an increase in force as shown in the experimental records of Fig. 3d and 5 ($-\text{Ca}^{2+}$). For an intermediate initial tension (Fig. 6c), the effects of detaching and reattaching cross-bridges partially cancel, producing a delay similar to the experimental traces of Figs 1b, 2a and 3c. At high starting tensions, a briefer delay before relaxation (simulated trace, Fig. 6a; experimental trace, Fig. 3a) largely represents the photochemical dark reactions (at 100 s^{-1}) between absorption of the UV radiation and ATP release. Reattached cross-bridges were assigned a fixed weight so the later phases of the transients, dominated by reattachments, did not depend on initial rigor tension. This superimposition of the tension traces in the final phases of relaxation is a natural consequence of the reattachment hypothesis and is difficult to reconcile with other

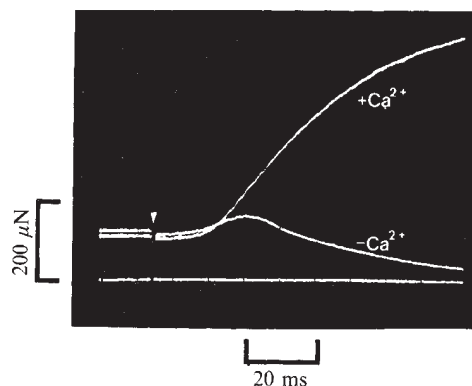


Fig. 5 Tension transients after liberation of $190 \mu\text{M}$ ATP in the presence ($+\text{Ca}^{2+}$) and absence ($-\text{Ca}^{2+}$) of free calcium. The muscle fibre (length 3.0 mm, sarcomere length $2.44 \mu\text{m}$, cross-sectional area $4.2 \times 10^{-9} \text{ m}^2$) was released by $30 \mu\text{m}$ 1 second before the laser pulse. Conditions were as described in Fig. 1 legend except that in the case of the $+\text{Ca}^{2+}$ trace, EGTA in the photolysis solution was replaced by 30 mM Ca-EGTA, to give a free Ca^{2+} concentration of $\sim 30 \mu\text{M}$.

possibilities. Although other properties of the filament lattice may contribute to the observed shape of the transients²¹, and the cross-bridge cycle in an active contraction may be kinetically very different, this model demonstrates that a cooperative attachment can explain the shape of the mechanical transients initiated by release of ATP within a rigor muscle fibre.

Discussion

The observed rate of detachment of rigor cross-bridges in our experiments is within an order of magnitude of the rate of actomyosin dissociation in solution. This indicates that detachment from the AM state is not greatly slowed by the structural constraints of the filament lattice. The value of the detachment rate constant determined from the rate of the final relaxation over a range of released ATP concentrations ($1.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) is probably an underestimate of the actual rate as the final decay apparently includes some slow components related to reattaching and cycling cross-bridges. One approach to determining the pure rate of detachment of the rigor cross-bridges is to consider that a mechanical stretch or release of the muscle influences the force production of a particular cross-bridge only until it detaches. In that case, the computed tension difference between two of the traces in the pre-stretch and pre-release experiment (Fig. 3) decays at a rate reflecting the first detachment of the original rigor bonds. The traces converge by 10–30 ms, whereas the final decay of force and stiffness takes longer than 100 ms. This suggests that in muscle fibres the ATP-induced detachment occurs about as rapidly as with the solubilized proteins^{3,7,13}.

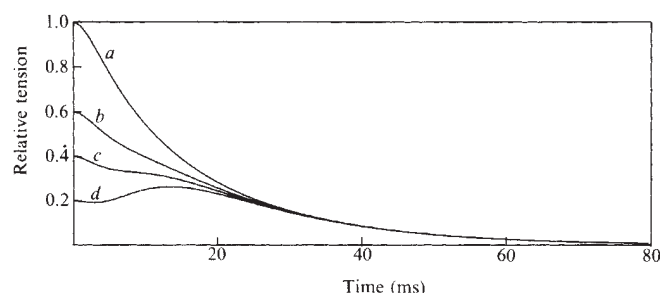


Fig. 6 Simulation of relaxation from rigor, including transient reattachment due to cooperativity between pairs of cross-bridges. Each cross-bridge of a pair was considered to be either in a rigor attachment, an active attachment or detached. Rigor states were further distinguished by whether they had existed continuously from the initiation of the simulation or whether they were formed from reattached active cross-bridges. In either case the second-order rate constant for detachment of a rigor cross-bridge by ATP was taken as $8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The reattachment rate was 50 s^{-1} for those detached cross-bridges that had an attached partner. If both sites of a pair were detached, then no further attachment was allowed for that pair. The rate constant for transformation of active cross-bridges to rigor-type attachments was 100 s^{-1} . No stress- or strain-dependence was considered for any of these rates. Initial rigor cross-bridge concentration was $200 \mu\text{M}$. To initiate the reaction, $500 \mu\text{M}$ ATP was introduced with a first-order rate constant of 100 s^{-1} . The resulting set of equations was integrated using the Gear method³⁷ to calculate how the concentration of each possible combination of pairs of cross-bridges varied with time. The forces generated by undetached rigor, active, and rigor bridges formed from active cross-bridges were given the following relative weights to generate curves of the simulated force transients after photolysis: a, 1, 3, 1; b, 0.6, 3, 1; c, 0.4, 3, 1; d, 0.2, 3, 1. Choice of these weights assumes that the state of reattached cross-bridges does not depend on the original rigor tension. Many features of the experimental records are apparent in this simulation.

Our experiments do not provide evidence concerning the extent of the direct hydrolysis pathway (dashed arrows in scheme 1) or the reversibility of the detachment step^{8,9}. However, when the strain on a cross-bridge, induced by an applied stretch, is relieved on the first detachment, then the rapid convergence of the tension traces discussed above indicates the rate of the first detachment of the cross-bridges whether or not subsequent reattachment occurs before the hydrolysis step.

Detachment of cross-bridges from the rigor state with a $t_{1/2}$ of less than 10 ms (Fig. 1) is strikingly faster than the steady turnover rate of the actomyosin ATPase in isometric contractions ($0.2\text{--}1 \text{ s}^{-1}$; refs 19, 22–24). Thus, as a cross-bridge proceeds through the isometric contractile cycle, only a small proportion of time (<1%) is spent in the AM state. If the AM state which is present in rigor muscle fibres is the same as the

nucleotide-free intermediate in the normal active cross-bridge cycle, then a cross-bridge which enters the AM state binds ATP and detaches within a few milliseconds.

During active shortening at forces less than the isometric value, the rate of energy liberation is three to five-fold higher than in an isometric contraction^{25–27}. This control of energy liberation is not expected to occur at the step of AM dissociation as that reaction is not rate-limiting. This was confirmed by experiments in which stretches and releases were applied to the fibres in rigor before the laser pulse (Fig. 3); there was no decrease of detachment rate when the tension was increased into the range of physiological contractions.

Force transients in single muscle fibres which are initiated by a rapid change in length also indicate that the mechanics of the rigor state are different from the major force-producing states populated in an active contraction^{28,29}. When an active muscle undergoes a small step decrease in length, the force drops and then recovers within the next few milliseconds to almost the original tension level³⁰. This quick recovery phase may reflect the normal force generation process. The structural correlate of the steps between attachment and force generation might be a rotation or rocking motion of the myosin head towards the Z-line^{2,30}. In rigor, force transients initiated by rapid length changes show much less quick tension recovery^{28,29}. Thus, the motions or configurations available to the cross-bridge in its force producing state are not all available to the rigor cross-bridge. Several previous studies of rigor cross-bridge orientation, using C-ray scattering³¹, fluorescence polarization³² and electron paramagnetic resonance³³, have indicated that alterations of force caused by applied length changes do not rotate rigor cross-bridges.

Which intermediates would be predominant in a contraction? Our experiments show that cross-bridge detachment is too fast for sites to spend a large proportion of time in the AM (rigor) state. Unless Ca^{2+} markedly slows detachment from rigor, the result in the presence of Ca^{2+} (Fig. 5) indicates that the reactions of the detached cross-bridge and of reattachment are also too rapid to be limiting in an active contraction cycle. These data suggest that the rate limitation occurs between force production by the cross-bridge and rebinding of ATP. During a steady active isometric contraction, most of the cross-bridges would then be attached in the states that generate force. X-ray and mechanical data also support the hypothesis that most cross-bridges are attached during active isometric contractions^{28,34}. The structural, mechanical, and kinetic data all suggest a model of the cross-bridge cycle in which the mechanical power transfer and control of energy liberation are separate from the detachment reaction and occur between cross-bridge attachment and the AM rigor intermediate.

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Functionally homologous cell cycle control genes in budding and fission yeast

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The cdc 2 (previously called wee 2) cell cycle start gene of Schizosaccharomyces pombe, which is required for start and the control of mitosis, has been isolated from an S. pombe gene bank by complementation of a cdc 2 mutation. A functionally homologous sequence which complements the cdc 2 mutation has also been isolated from a Saccharomyces cerevisiae gene bank and this sequence has been shown to contain the cdc 28 cell cycle start gene of S. cerevisiae. It is concluded that the cdc 2 and cdc 28 genes perform homologous cell cycle control functions in the two organisms.

THE fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* have both been used as model organisms for studying the eukaryotic cell cycle^{1,2}. This work has made extensive use of temperature-sensitive *cdc* (cell division cycle) mutants which become specifically arrested during the cell cycle when incubated at their restrictive temperatures; over 40 *cdc* genes have been described in *S. cerevisiae* and over 25 in *S. pombe*. All of these are required for normal cell division but only a few are expected to be involved directly in cell cycle controls such as commitment to the mitotic cycle and regulation of the rate of cell division. In *S. cerevisiae*, four genes (*cdc 28*, *36*, *37* and *39*) have been shown to have roles in a major cell cycle control called 'start'³⁻⁶. Cells accumulate in G₁ at start in poor nutritional conditions and under the influence of the mating hormones. Mutants of the four *cdc* genes block cell cycle progress at start and are able to conjugate from their point of cell cycle arrest. Once start has been completed the cell becomes committed to the mitotic cycle and is unable to undergo the alternative developmental pathway of conjugation. Completion of start is also the major rate-limiting step of the cell cycle regulating the rate of cell division^{3,4}. In *S. pombe* there are two start genes, *cdc 2* and *cdc 10* (ref. 7). Mutants of these two genes block in G₁ and are able to conjugate from that point of arrest. In poor nutritional conditions, *S. pombe* cells also accumulate before start. Thus the cell cycles of both organisms can be divided operationally into an uncommitted pre-replicative phase before start and a committed replicative phase consisting of S-phase, mitosis and cell division after start (Fig. 1).

Although start divides the cell cycles of the two yeasts into two similar phases this does not necessarily mean that the two start events have the same molecular basis in the two organisms, especially as they are not closely related. To establish whether the molecular basis of start is indeed similar we have made use of the fact that *cdc* start genes can be physically isolated by complementation and can be transferred from one yeast to the other. From these experiments we establish that *S. cerevisiae cdc 28* can complement *cdc 2* mutations of *S. pombe*, suggesting that the start events have a similar molecular basis in the two organisms.

Isolation of the *S. pombe cdc 2* gene

A sequence containing a *cdc* start gene of *S. cerevisiae* has been selected from a bank of budding yeast DNA by its ability to complement the appropriate *cdc* mutation⁸. The development of techniques for high frequency transformation of *S. pombe* and the construction of a gene bank in a yeast-bacterial shuttle vector^{9,10}, makes the same approach feasible in *S. pombe*.

A *Hind*III partial *S. pombe* gene bank was made in vector pDB262, which contains the *S. cerevisiae leu 2* gene and can complement the *leu 1.32* mutation of *S. pombe*^{9,10}. The gene bank was used to isolate the *cdc 2* start gene by transforming a *cdc 2.33 leu 1.32* strain to Leu⁺ prototrophy and replica-plating the resultant 30,000 clones to 35 °C, at which temperature *cdc 2.33* cells elongate and die (Fig. 2b; ref. 11). Clones that continued to divide at 35 °C were isolated and the plasmids which they contained were recovered in *Escherichia coli*^{9,10}. One of these plasmids was found to complement the *cdc 2.33* and *cdc 2.M26* mutations when retransformed back into strains containing these mutations. The dividing cells in these transformant clones were elongated (Fig. 2c) compared with wild-type dividing cells (Fig. 2a), indicating incomplete suppression of the mutant phenotype by the insert on the plasmid. The plasmid was termed pcdc2.3(Sp) and contained a 7-kilobase (kb) insert. A 1.4-kb *Hind*III fragment from this insert was recloned into vector pDAM6 which contains the *S. cerevisiae leu 2* and pBR322 but no sequence capable of supporting replication in *S. pombe*. This plasmid, pcdc2.32(Sp), was transformed into a *S. pombe leu 1.32h⁻* strain and some of the Leu⁺ prototrophic transformants formed were found to be mitotically stable and were presumed to have arisen by plasmid integration into the chromosomal *cdc 2* gene. This was confirmed for two of the integrants by preparing DNA from cells of the *leu 1.32h⁻* strain before integration and after integration of pcdc2.32(Sp). The DNA was digested with *Bam*HI, an enzyme which does not cut within the 1.4-kb *Hind*III insert but cuts once in the vector pDAM6 (Fig. 3). After Southern transfer the digested DNAs were probed with ³²P-labelled pcdc2.32(Sp) (Fig. 3). The 13-kb *Bam*HI fragment of DNA from cells before integration was altered to two fragments of 17 and 8 kb after integration for both integrants (see Fig. 3 for explanation). This result demonstrates that pcdc2.32(Sp) integrated at the chromosomal site homologous to the 1.4-kb *Hind*III insert. We demonstrated that this site was the *cdc 2* locus by showing that the Leu⁺ marker phenotype of the plasmid was closely linked to *his 3*, a marker localized within 1 centimorgan of the *cdc 2* locus¹². The two pcdc2.32(Sp) integrants in strain *leu 1.32h⁻* were crossed to a *his 3⁻ leu 1.32h⁺* strain. The resultant zygotes were sporulated and the phenotypes of 400 spores of both crosses examined. In both cases less than seven recombinant spores (*leu 1⁺ his 3⁻* or *leu 1⁻ his 3⁺*) were observed, indicating that the *S. cerevisiae leu 2* gene must have integrated within 1.7 centimorgans of *his 3*. Therefore, pcdc2.3(Sp) contains the *S. pombe cdc 2* start gene.

Isolation of a *S. cerevisiae* sequence complementing *cdc 2*

Having shown that a sequence containing the *cdc 2* gene could be isolated from *S. pombe*, we next tried to establish whether

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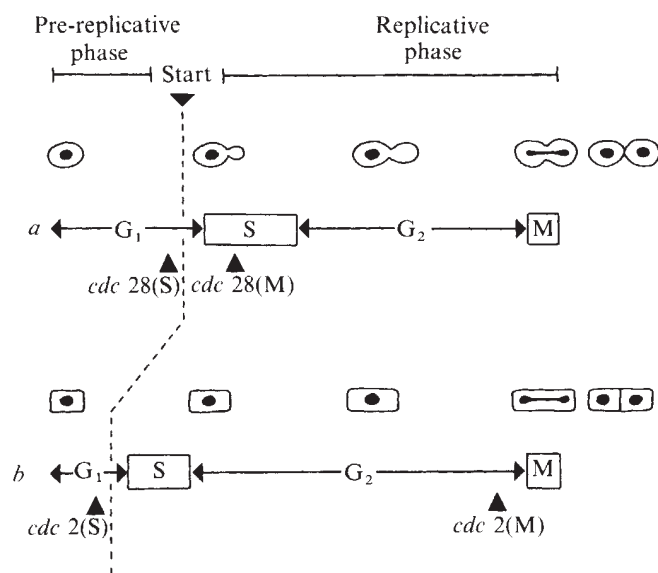


Fig. 1 Schematic representation of cell cycle control in *S. cerevisiae* (a) and *S. pombe* (b). Start divides the two cycles operationally into an uncommitted pre-replicative phase and a committed replicative phase. The pre-replicative phase occupies most of G₁. It is the major expandable part of the cycle, becoming much longer at slow growth rates. The figure represents rapidly growing cells in which the pre-replicative phase is longer in *S. cerevisiae* than in *S. pombe*. The transition or execution points of *cdc 28* and *cdc 2* are shown as filled triangles, the ones appropriate for start are labelled *cdc 28*(S) and *cdc 2*(S) and those appropriate for mitosis are *cdc 28*(M) and *cdc 2*(M).

there are any sequences in *S. cerevisiae* which have the same function as the *cdc 2* gene. This can be done by introducing a *S. cerevisiae* gene bank into *S. pombe* and testing for complementation of the *cdc 2.33* mutation. Previously, such complementation has only been observed for genes involved in intermediary metabolism such as *leu 2* (refs 9, 10). A *S. cerevisiae* gene bank has been constructed in YEp13 (provided by K. Nasmyth), a vector which contains the *leu 2* gene and can autonomously replicate in *S. pombe*. This gene bank was used to transform the *S. pombe* strain *cdc 2.33 leu 1.32* in a manner identical to that described above for the isolation of *pcdc2.3*(Sp). A plasmid, *pcdc2*(Sc), was isolated which complemented the *cdc 2.33* and *cdc 2.M26* mutations. This plasmid contained *S. cerevisiae* sequences as its insert (data not given)

and generated *cdc 2*⁺ transformants having the same elongated appearance at the restrictive temperature as the *S. pombe* *pcdc2.3*(Sp) (Fig. 2c). Thus, *S. cerevisiae* contains DNA sequences which can functionally complement mutations in the *cdc 2* gene.

S. cerevisiae cdc 28 can complement *cdc 2*

As *cdc 2* is a start gene in *S. pombe*, *pcdc2*(Sc) might contain one of the four known *S. cerevisiae* start genes^{5,6}. All of these have been isolated in the plasmids *pcdc28*, 36, 37 and 39 (ref. 8 and S. Reed, unpublished results) using vector YRp7 or its derivative YRp7'. The four plasmids were digested with *Hind*III and after Southern transfer were probed with ³²P-*pcdc2*(Sc). The hybridization observed in this experiment indicated that the inserts of *pcdc2*(Sc) and *pcdc28* (containing the *cdc 28* gene) contained common sequences. In particular, both plasmids had a 2.3-kb *Hind*III fragment in common. To test this was the same sequence, the 2.3-kb *Hind*III fragment was recovered from a digest of *pcdc28*, then nick-translated and hybridized to the similarly sized fragment from *pcdc2*(Sc) (Fig. 4). As the *pcdc28* sequences are unique in the *S. cerevisiae* genome (S. Reed, unpublished results) this hybridization establishes that *pcdc2*(Sc) contains *cdc 28* sequences. To confirm that *pcdc2*(Sc) contains a functional *cdc 28* gene, the plasmid was transformed into a *cdc 28.4 leu 2.3 S. cerevisiae* strain (provided by I. Johnson). The resultant *Leu*⁺ prototrophs grew at 36°C, the restrictive temperature of *cdc 28.4*, demonstrating that *pcdc2*(Sc) can complement a *cdc 28* mutation and thus contains a functional *cdc 28* gene. These experiments show that the *cdc 28* gene contained within *pcdc2*(Sc) can complement mutations of *cdc 2*. To test whether the *cdc 28* gene contained within *pcdc28* will also complement mutations of *cdc 2*, the *pcdc28* sequences first had to be transferred to a different vector because YRp7 cannot be selected for in *S. pombe*; *pcdc28* was partially digested with *Sau*3A and ligated with *Bam*HI-digested pDB248. The crude ligation mix was used to transform the *cdc 2.33 leu 1.32 S. pombe* strain to *Leu*⁺ prototrophy. About 30% of the transformants were able to form colonies at the restrictive temperature of 35°C. The cells of these transformants were smaller (Fig. 2c) than wild-type cells (Fig. 2a) and were very similar to certain *wee* mutant alleles of *cdc 2* (Fig. 2d). These *wee* mutant alleles have been interpreted as having an abnormally high *cdc 2* gene product activity. Note that the behaviour of *pcdc28* in *S. pombe cdc 2.33* strains differs from that of *pcdc2*(Sc), which resulted in weaker complementation and the formation of elongated

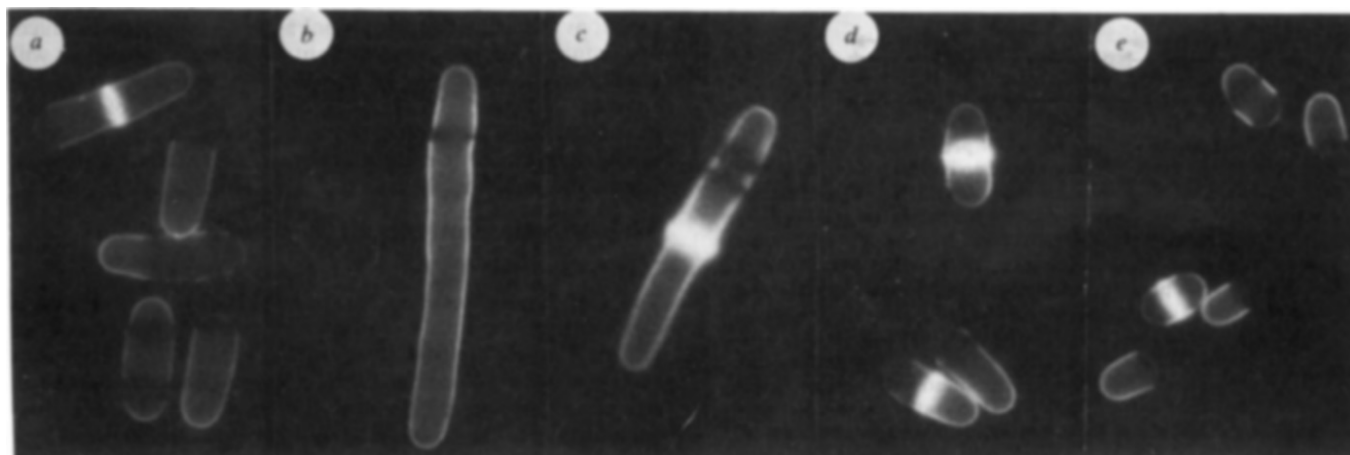


Fig. 2 Cells of *S. pombe* strains stained with Calcofluor and photographed by fluorescence microscopy. In these conditions the septa of dividing cells fluoresce intensely. a, Wild-type 972; b, *cdc 2.33 leu 1.32*; c, *cdc 2.33 leu 1.32* containing *pcdc2.3*(Sp); d, *cdc 2.1w* (previously called *wee 2.1*); e, *cdc 2.33 leu 1.32* containing *pcdc28* in pDB248. Cells were grown in minimal medium at 25°C overnight and were transferred to 35°C in yeast extract medium for 7 h before staining as described in ref. 11. The cells in e include some dividing at a small size, similar to *cdc 2.1w*, and others which are highly elongated such as *cdc 2.33* which have lost the plasmid and are unable to divide. *pcdc2.3*(Sp) is based on vector pDB262 which contains part of the yeast 2 μm circle, *leu 2* and the bacterial plasmid pTR262. pDB248 is similar but with pTR262 replaced by pBR322^{9,10}.

cells (Fig. 2c). It is unknown why these two plasmids differ in their behaviour but as they were located in different vectors and were isolated from different gene banks the plasmid copy number and the flanking chromosomal and vector sequences could be different. Either of these two factors could influence the final activity of the *cdc 28* gene product in *S. pombe*.

S. pombe cdc 2 cannot complement *cdc 28*

As *cdc 28* can complement *cdc 2* mutations of *S. pombe*, it was of interest to determine whether *cdc 2* could complement *cdc 28* mutations of *S. cerevisiae*. This was tested by transforming the *S. cerevisiae* strain *cdc 28.4 leu 2.3* to *Leu*⁺ prototrophy using *pcdc2.3*(Sp). The *Leu*⁺ transformants did not grow at 36 °C, forming enlarged cells that were both budded and unbudded. This failure to complement indicates that the *S. pombe cdc 2* gene cannot substitute for a defective *cdc 28* gene in *S. cerevisiae*, which could be due to inadequate *cdc 2* expression or inability of the *cdc 2* gene product to provide the entire function of *cdc 28*. It has been shown that the 5' regulatory sequences of the cytochrome *c* and alcohol dehydrogenase genes differ in *S. pombe* and *S. cerevisiae* (ref. 13 and P. Russell, personal communication). Thus the 5' sequences of *cdc 2* may be unable to initiate transcription at a sufficient level in *S. cerevisiae* to complement mutations of *cdc 28*.

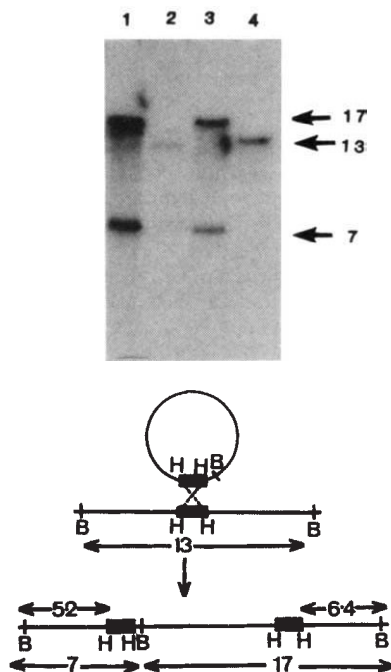


Fig. 3 A model of the integration of *pcdc2.32*(Sp) into the *cdc 2* site on chromosome II (bottom) and an insert showing Southern blot transfers of *S. pombe* chromosomal DNA digested with *Bam*HI (top) prepared from: tracks (1) and (3), *pcdc2.32*(Sp) integrants; tracks (2) and (4), the *leu 1.32* strain before integration. B, *Bam*HI sites; H, *Hind*III sites. All procedures for DNA preparation, nick-translation with ³²P-CTP and Southern blot transfers are described in refs 9, 10. The fragments produced by integration can be explained as follows. The 1.4-kb *Hind*III fragment (shown as a thick line) derived from *pcdc2.32*(Sp) is contained within a 13-kb *Bam*HI chromosomal fragment. When *pcdc2.32*(Sp) of total length 11 kb integrates via homologous recombination at the *cdc 2* site, a tandem duplication of the 1.4-kb *Hind*III fragment is formed which flanks the vector sequences of pDAM6. As *pcdc2.32*(Sp) contains only one *Bam*HI site situated in the vector next to the chromosomal insert, two new *Bam*HI fragments are generated with a combined length of 24 kb made up of the original *Bam*HI chromosomal fragment of 13 kb and *pcdc2.32*(Sp) (11 kb). The precise sizes of the two *Bam*HI fragments generated can be explained if it is assumed that the 1.4-kb *Hind*III fragment is located 5.2 and 6.4 kb from the chromosomal *Bam*HI sites.

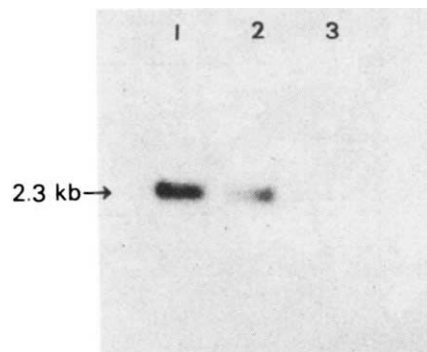


Fig. 4 Southern blot transfers of DNAs digested with *Hind*III and probed with the 2.3-kb *Hind*III fragment derived from *pcdc28* (see text). Track (1), *pcdc28*; (2), *pcdc2*(Sc); (3), YEp 13. Note that a single 2.3-kb fragment hybridizes in *pcdc28* and *pcdc2*(Sc) but there is no hybridization with YEp 13. All procedures for DNA preparation, nick-translation with ³²P-CTP and Southern blot transfers have been described elsewhere^{9,10}.

cdc 2 and *cdc 28* genes perform similar functions in both G₁ and G₂

The requirement for the *S. pombe cdc 2* gene in G₁ at start has already been described; *cdc 2* is also required in G₂ before mitosis (Fig. 1). Whether cells become blocked in G₁ or G₂ depends on their position in the cell cycle at the time of temperature shift⁷. It has recently been shown that certain *cdc 28* mutants of *S. cerevisiae* also arrest in G₂ just before mitosis as well as in G₁ at start¹⁴. Thus *cdc 28* also has a dual role in the cell cycle at start and at mitosis, and is presumably providing both these functions when complementing *cdc 2* mutations of *S. pombe*. This strongly suggests that the molecular function of the *cdc 2* and *cdc 28* genes is the same or very similar. To determine whether the *cdc 2* and *cdc 28* sequences are also closely related, *pcdc2.3*(Sp) was digested with *Hind*III and after Southern transfer was hybridized with ³²P-*pcdc2*(Sc). No hybridization of the inserts was detected in normal stringency conditions (0.75 M NaCl, 65 °C). This lack of sequence homology is not surprising as the two yeasts are not closely related and no hybridization was observed between *S. pombe leu 1* and *S. cerevisiae leu 2* genes, both of which encode the same enzyme, β-isopropylmalate dehydrogenase^{9,10}. DNA sequencing will be required to establish the precise relationship between *cdc 2* and *cdc 28*.

The molecular mechanism of the *cdc 2* and *cdc 28* gene functions at start and mitosis is unknown. As the gene products are required for two very different cell cycle events, they will possibly have some sort of triggering coordinating role. We envisage a molecular function such as that of protein modifications in the control of many other cellular processes¹⁵, modulating different molecular activities at start and mitosis, but via the same mechanism.

The mitotic controls in *S. pombe* and *S. cerevisiae*

The *cdc 2* gene function has been shown to be the major rate-limiting step in *S. pombe*, determining the timing of mitosis^{16,17}. *wee* mutant alleles at the *cdc 2* locus result in a cell cycle having a shorter G₂ than normal and a reduced cell size at mitosis and cell division. Before mitosis can occur, the cell must grow to a critical size and this requirement is reduced in the *wee* mutants^{25,26}.

In *S. cerevisiae* there is no mitotic control analogous to that found in *S. pombe*. Mitosis is thought to occur when a certain time has elapsed after 'start'^{18,19}. The difference in control between the two yeasts may be related to the fact that mitosis is apparently initiated very early in the cell cycle in *S. cerevisiae*. A short intranuclear spindle forms by the end of G₁ (ref. 20). This structure elongates later in the cycle to form the full mitotic

spindle during mitosis. The requirement of *cdc 28* for mitosis is also completed early, about one-tenth of a cell cycle after start (Fig. 1; ref. 14). These observations suggest that the initiation of mitosis occurs early in the cell cycle in *S. cerevisiae*, a situation which is different from most eukaryotic cells such as *S. pombe*. When *cdc 28* is introduced into *S. pombe* as *pcdc28*, mitosis is initiated prematurely so that cell division occurs at a small size (Fig. 2e). One explanation for this could be that the *cdc 28* gene lacks some of the regulatory functions of *cdc 2* which ensure that mitosis is delayed until the *S. pombe* cell has grown to normal size. *cdc 28* may not have these functions because this control is not normally operative in *S. cerevisiae*.

Cell cycle control in other organisms

The *cdc 2* and *cdc 28* genes have major roles in the cell cycle control of *S. pombe* and *S. cerevisiae* and must perform identical

or similar molecular functions. But the two organisms are not closely related as judged by both taxonomic criteria²¹ and the comparison of 5S RNA²² and cytochrome *c*²³ sequences, which show <70% homology. Despite this considerable evolutionary divergence, the molecular mechanisms of start and of the control initiating mitosis appear to have been highly conserved, thus we believe that these control mechanisms may also be applicable to other eukaryotes. In mammalian cells, G₁ restriction or transition points analogous to start in the yeasts have been proposed as the major point of cell cycle control²⁴. Thus it is possible that the control in mammalian cells may also involve a functional equivalent of the *cdc 2* and *cdc 28* gene products.

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Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing γ , ϵ and α genes

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Cosmid clones containing the human γ , ϵ and α heavy chain constant region genes and an ϵ pseudogene have been isolated. All these genes have a switch sequence detectable by hybridization. We have studied overlapping cosmids covering two separate regions of the genome, and the gene order in each of these regions was found to be γ - γ - ϵ - α . This implies an evolutionary duplication in this multigene family involving γ , ϵ and α genes.

MOST known mammalian genes are organized in multigene families whose members share sequence homology. Such families include the globin genes^{1,2}, interferon genes³, growth hormone genes⁴, genes of the major histocompatibility complex⁵ and immunoglobulin genes. The immunoglobulin heavy (H) chain genes consist of four linked families of gene segments: the variable (V_H), diversity (D), joining (J_H) and constant (C_H) gene segments. The C_H gene segments determine the class of the H-chain polypeptide. Both man and mouse have five classes of H chain, known as μ , δ , γ , ϵ and α , which differ in their biological activities. In both species the γ chains are further divided into four subclasses, and in man there are two subclasses of α chain. When the active H-chain gene is formed by V_H/D/J_H integration^{6–8} the C_H gene closest to the J_H locus, the μ gene, is the first to be expressed while the other C_H genes can subsequently be expressed by H-chain switching mechanisms. One of these mechanisms, which operates for each C_H gene except δ , involves deletion of all C_H genes between the V gene and the newly expressed C_H gene^{9–12}. This switch deletion has

been proposed to involve recombination, perhaps by unequal sister chromatid exchange^{13–15}, between homologous, internally repetitive sequences (called switch or S regions) which were found upstream of each mouse C_H gene except δ ^{14,16–18}. It was similarly found in man that the μ gene S region has a repetitive sequence and is homologous with a region upstream of γ 2 but not δ ¹⁹. Comparison of S regions of mouse μ , γ and α genes with their human counterparts has shown strong evolutionary conservation^{19–22}.

The order of the C_H genes in the mouse has been determined as 5'- μ - δ - γ 3- γ 1- γ 2b- γ 2a- ϵ - α -3', and no pseudogenes have been reported¹⁸. The existence of two α -chain subclasses in man and the finding that human DNA contains a pseudo γ gene ($\psi\gamma$)^{22,23} and three ϵ -like genes^{24–26} implied that the arrangement of human C_H genes was different from that in the mouse. Some aspects of the arrangement of human C_H genes have already been published. The J_H- μ - δ region was mapped and found to be organized similarly to the mouse^{19,27}, the γ 2 and γ 4 genes were shown to be about 19 kilobases (kb)

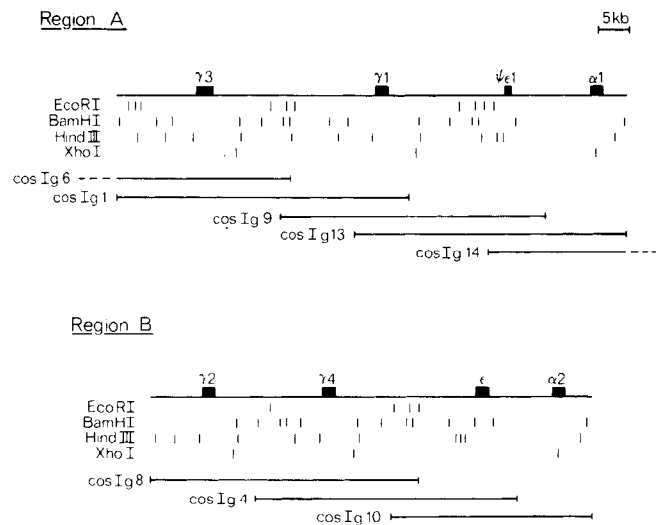


Fig. 1 Restriction maps of two regions of human DNA covered by overlapping cosmid clones. The cosmids were digested with the four enzymes shown, singly and in all pairwise combinations. Restriction fragments were separated on 0.8% agarose gels, and after reducing the fragment sizes by treatment with acid and then base³⁷, the DNA was blotted on up to four filters sequentially³⁸. Each filter was hybridized to one of four probes: γ , ϵ or α gene probes as described in the text or the cosmid vector pOPF1 (F. Grosveld, personal communication). Overlaps between cosmids were confirmed by running all corresponding digests of overlapping clones in adjacent slots of agarose gels. The ends of cosIg6 and cosIg14 which extend beyond region A are shown as dotted lines because the restriction map in these regions has not been confirmed by comparison with other cosmid or phage λ clones.

apart^{22,23,28}, and it was reported that the ϵ and $\psi\epsilon 1$ genes are each linked to an α gene²⁴. The $\psi\epsilon 2$ gene is apparently a processed pseudogene which has lost its intervening sequences, has a poly(A)-rich tail and has been conveyed to a new chromosomal location²⁴. We wanted to study the organization of the human C_H genes further both to allow an evolutionary comparison with the mouse and also to allow a comparison of the H-chain genes of normal humans with those of individuals with immune deficiencies or leukaemias such as Burkitt's lymphoma. We present here a partial characterization of the C_H gene locus in man. Cosmid clones were isolated which apparently contained all the expressed γ , ϵ and α genes and $\psi\epsilon 1$. All of these genes have switch sequences which hybridize to S_{μ} . By mapping overlapping cosmid clones we identified two separate blocks of C_H genes, each with the order γ - γ - ϵ - α , implying an evolutionary duplication involving γ , ϵ and α genes. We propose an order of these two blocks of genes relative to each other and relative to the $J_H/\mu/\delta$ region.

Isolation and restriction mapping of cosmids containing γ , ϵ and α genes

Approximately 5×10^5 colonies of a human genomic cosmid library (F. Grosveld, personal communication) were screened with human γ and ϵ C_H gene probes. The γ probe was the 3.6-kb *EcoRI*-*HindIII* insert of p3.6RH4.2 (ref. 23) which contains the whole $\gamma 3$ gene, while the ϵ probe was the 2.0-kb *BamHI*-*PstI* insert of $\epsilon 1.2$ BP25 (ref. 25) which contains an entire ϵ gene. Several cosmid clones were isolated with these probes. Restriction mapping showed that the cosmids fall into two separate groups of overlapping clones, each group representing an unbroken region of chromosomal DNA, termed here region A and region B (Fig. 1). γ , ϵ and α genes were found in each region and located within the regions by hybridization to the γ or ϵ probes described above or to a mouse α cDNA probe consisting of the 0.6-kb *EcoRI* fragment from the 3' end of the insert of p107 α R5 (ref. 29). Both regions A and B were found to have a gene order γ - γ - ϵ - α . The total sizes of

regions A and B are approximately 83 and 72 kb, respectively, and the distance between genes within each region ranges from 10 to 26 kb, which is similar to the spacing found between mouse γ , ϵ and α genes¹⁸. It is unlikely that the cosmids have undergone deletions because all parts of region A are covered by at least two cosmids, and although the terminal areas of region B are covered by only one cosmid, our map in those areas is corroborated by published maps of phage λ clones^{22-26,28}.

Characterization of genes present in cosmid clones

We obtained evidence by restriction mapping and hybridization as described above for four γ , two ϵ and two α class genes in the cosmids which we isolated. These genes were assigned to subclasses by sequencing or, in the case of some of the genes for which sequences were already available, by restriction mapping. However, we have not yet fully sequenced any of the genes present in these cosmids, so the subclass assignments remain provisional.

Restriction maps and partial sequences were known which distinguished the four γ subclass genes and the $\psi\gamma$ gene (U. Krawinkel and T.H.R., unpublished results and refs 22, 23, 28), so we could identify the γ genes in the cosmids on the basis of the restriction maps shown in Fig. 1. Apparently, the cosmids contain the $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ genes, but not $\psi\gamma$. In the case of the $\gamma 1$ and $\gamma 3$ genes, this identification was confirmed by sequencing their distinctive hinge regions. Each hinge coding segment begins with a *PstI* site²³, so *PstI* fragments of cosIg1 and cosIg13 were subcloned into M13mp9 (ref. 30) and subclones containing hinge region fragments were selected by hybridization with the 1.8-kb *PstI* fragment of p3.6RH4.2, which contains the fourth hinge of $\gamma 3$. These clones were sequenced by the chain terminator method³¹, giving sequences which agree with those previously published for the $\gamma 1$ hinge coding segment and the four hinge coding segments of $\gamma 3$ (data not shown). Identical $\gamma 1$ hinge region sequences were obtained from cosIg1 and cosIg13, confirming the overlap between these two clones.

The ϵ gene in region B (Fig. 1) was identified as the active gene by restriction site comparison with the expressed ϵ gene of an IgE-producing myeloma, which is contained in the λ clone $\lambda\epsilon 1.2$ (ref. 25). Southern blot hybridization using the ϵ probe $\epsilon 1.2$ BP25 showed that cosIg4, cosIg10 and $\lambda\epsilon 1.2$ have identical

Pseudoepsilon 1

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AAGCTTAGCTGGTTGGGCTGAGTAAGCTGGGCTGAGCTAAATGGGATTGAGCTGAGGAGG 60
GCTAGGCTGGGGGAGAGACCTGACGACGAGAGGGTTAAAGCTGGAGTGAGCAGGCCCTT 120
AAATTATTGAACATAATTGGGCTGGGGTGATCTGAATTTAGCTGGGATGAGCTGGGCTGG 180
GCTGAACCTGTGCCACGTGAACCTGGGCTAAACTAGGCTGCGCTGAGTGGAGCTGAGCTGG 240
TTGGTCTCAACTGGGTTCAAGCTGAGCTGGGCTGCGCTGAGTGGAGCTGAGCTGAG 300
ACTACACTGGGTTCAACCCGAGAGGGGTGAGCGCTACCTAAGCCGGCCAGCCGCTTC 360
N P R G V S A Y L S R P S P F D
ACTACACTGGGTTCAACCCGAGAGGGGTGAGCGCTACCTAAGCCGGCCAGCCGCTTC
L F I
ACCTGTTCAAT

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Fig. 2 Partial sequence of the $\psi\epsilon 1$ gene. *HindIII*-*BamHI* fragments of cosIg13 were subcloned in M13mp8³⁰, and a subclone containing the 2.0-kb *HindIII*-*BamHI* fragment with ϵ -like sequences was selected by hybridization with the insert of $\epsilon 1.2$ BP25. This subclone was sequenced from the *HindIII* site by the chain terminator method³¹, giving the sequence shown above. The region of the sequence which is identical to the active ϵ gene is underlined and surmounted by a deduced amino acid sequence in the single letter code.



Fig. 3 Partial sequences of the putative $\alpha 1$ (a) and $\alpha 2$ (b) genes. c, Southern blot comparing α genes in cosIg10, cosIg13 and placental DNA. **Methods:** The 3.2-kb *XhoI*-*HindIII* fragments of cosIg10 and cosIg13 were subcloned in M13mp9³⁰ and sequenced from the *XhoI* site by the chain terminator method³¹. The deduced amino acid sequence is shown in the single letter code above the nucleotide sequence, and an arrow marks the splice site at the end of the C_H2 domain. Residues known to differ between the $\alpha 1$ and $\alpha 2$ proteins are boxed³². In c, all the DNAs were digested with *PstI*, fractionated on a 1.4% agarose gel and blotted onto a nitrocellulose filter³⁹. The DNA was then hybridized with 2×10^6 c.p.m. per ml of nick-translated $\alpha 2XP8$ at 65 °C with a salt concentration of $1 \times SSC$ and washed at 65 °C with $0.1 \times SSC$ ⁴⁰. The filter was then exposed to preflashed film at -70 °C with an intensifying screen⁴¹.

sites for *Bam*HI, *Pst*I, *Sal*I, *Bgl*II, *Sma*I, *Pvu*II and *Hinc*II, at a total of nine sites distributed in and to either side of the ϵ gene. This result confirms the overlap between cosIg4 and cosIg10 and indicates that these cosmids contain the active ϵ gene which has been sequenced previously^{24,25}.

The ϵ gene detected by hybridization in region A (Fig. 1) was found by nucleotide sequencing to be an unusual type of pseudogene. Hybridization of the insert of $\epsilon 1.2BP25$, which contains all four coding segments of the active ϵ gene, was confined to a 2.0-kb *HindIII*-*Bam*HI fragment of cosIg13. This fragment was subcloned in M13mp8 (ref. 30) and sequenced, by the chain terminator method, 374 bases from the *HindIII* site (Fig. 2). For the first 313 bases this sequence shows homology to the S_μ region repeats but thereafter it is identical with the sequence of the active ϵ gene, except that it starts four bases into the $C_\epsilon 3$ coding segment. The $C_\epsilon 3$ segment therefore lacks an acceptor RNA splice site. We obtained other sequences homologous to $C_\epsilon 3$ and $C_\epsilon 4$ of the active ϵ gene by sequencing *Hpa*II fragments of cosIg13 (data not shown). Although the ϵ gene in cosIg13 contains $C_\epsilon 3$ and $C_\epsilon 4$ sequences, we found no evidence of $C_\epsilon 1$ or $C_\epsilon 2$ sequences by hybridization to the $\epsilon 1.2BP25$ probe. This gene thus seems to be a pseudogene which has lost the first two coding segments of an active ϵ gene by a deletion which fused an S-like sequence to a site just inside the $C_\epsilon 3$ coding segment. This deletion is apparently not a cloning artefact because the $\epsilon 1.2BP25$ probe hybridizes to a *HindIII*-*Bam*HI fragment of the same size in human genomic DNA and in cosIg13 (data not shown). Similar information was recently reported from studies on a phage λ clone, apparently describing the same ϵ pseudogene, which was called $\psi\epsilon 1$ (ref. 24). The structure of the $\psi\epsilon 1$ gene is strikingly analogous to the mouse myeloma IF2 mutant gene in which the internally repetitive switch sequences were first noted¹⁴. In both the $\psi\epsilon 1$ gene (which is a germ-line mutation) and the IF2 mutant (which is an *in vitro* mutant of a $\gamma 1$ -secreting myeloma cell line), sequences which are homologous to the S region have been joined to a site within a gene. In the former case this site is just within a coding region while in the latter it is within an intervening sequence, just before a coding region. Evidently, aberrant rearrangements involving S sequences are important in the formation of these mutations.

Preliminary identification of the α genes present in cosmid groups A and B was achieved by partial nucleotide sequencing and comparison of the derived amino acid sequences with the known $\alpha 1$ and $\alpha 2$ protein sequences. The mouse α probe described earlier (which contains only $C_{\alpha 2}$ and $C_{\alpha 3}$ sequences)

hybridizes to a 3.2-kb *XhoI*-*HindIII* fragment of both cosIg13 (region A) and cosIg10 (region B). This fragment of each cosmid was subcloned in M13mp9 and more detailed restriction mapping of the subclones showed that in each case the *XhoI* site is within the α gene. The M13 subclones were therefore sequenced from the *XhoI* site towards the *HindIII* site, yielding the sequences shown in Fig. 3a and b. These sequences cover almost all of the $C_{\alpha 2}$ domain, over a region where the $\alpha 1$ and $\alpha 2$ protein sequences are known to differ at seven characteristic amino acid residues³². Our deduced amino acid sequences for the genes in cosIg13 and cosIg10 correspond exactly to published protein sequences for $\alpha 1$ and $\alpha 2$, respectively³², except for differences or ambiguities of amide assignment which are probably due to technical problems of protein sequencing. It therefore seems likely that the α gene in cosIg13 is $\alpha 1$ and that in cosIg10 is $\alpha 2$.

To confirm the hypothesis that cosIg13 and cosIg10 contain the active $\alpha 1$ and $\alpha 2$ genes we examined the number of α genes in human genomic DNA by Southern filter hybridization. Figure 3c shows a Southern blot comparing placental genomic DNA, cosIg13 and cosIg10, all digested with *Pst*I. The blot was hybridized to subclone $\alpha 2XP8$, which contains a 0.6-kb fragment of cosIg10 extending from the *XhoI* site near the start of $C_{\alpha 2}$ to a *Pst*I site in $C_{\alpha 3}$. The genomic DNA shows only two bands: a 2.0-kb band co-migrating with the band from cosIg10 and a 1.2-kb band co-migrating with the band from cosIg13. As no other hybridizing bands were detected, it seems likely that only two α genes exist in the genome and that the genes which we have cloned and partially sequenced are the active $\alpha 1$ and $\alpha 2$ genes. However, we cannot rule out the possibility that each band detected by Southern blotting in genomic DNA represents more than one gene. This caveat also applies to the γ and ϵ genes.

All the γ , ϵ and α genes have S regions homologous to S_μ

The H-chain class switch in antibody secreting cells is believed to be mediated by recombination between homologous S sequences near each C_H gene except δ . If this is correct we would expect to find that the human μ , γ , ϵ and α genes have homologous S sequences. We have studied this possibility by hybridization of an S probe, derived from the human μ gene, with human γ , ϵ and α genes. Figure 4 shows a Southern blot hybridization in which a restriction fragment containing S_μ was used to probe cosmids containing all the γ , ϵ and α genes described here and $\psi\epsilon 1$. As expected, all these genes, including

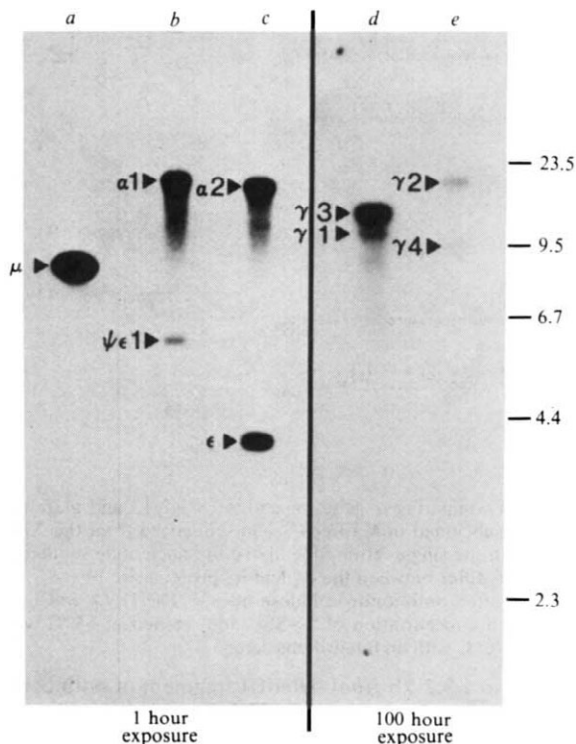


Fig. 4 Southern filter hybridization between an S_{μ} probe and S regions of other C_H genes. The probe was a 5.0-kb fragment of C76R51 (a subclone which contains the 8-kb *Eco*RI fragment from the 5' end of λ C76¹⁹) extending from an *Eco*RI site 45 base pairs inside the μ gene to a *Hind*III site in the 5'-flanking region of the μ gene. The restriction digests were as follows: a, $\cos$ Ig1 $\times$ *Bam*HI; b, $\cos$ Ig8 $\times$ *Bam*HI and *Eco*RI; c, $\cos$ Ig10 $\times$ *Bam*HI; d, $\cos$ Ig13 $\times$ *Bam*HI; e, C76R51 $\times$ *Eco*RI. Restriction fragments were separated on a 0.8% agarose gel and blotted onto a nitrocellulose filter³⁹. The filter was then hybridized with 2×10^6 c.p.m. per ml of nick-translated probe in low stringency conditions: the hybridization and all washes were at 65 °C at a salt concentration of $6 \times \text{SSC}^{40}$. The filter was then autoradiographed at -70 °C with an intensifying screen⁴¹ for the times indicated.

$\psi\epsilon_1$, have an S sequence which hybridizes to S_{μ} . This observation is consistent with a role for the S sequences in H-chain switching. However, even in the conditions of low hybridization stringency used here, S_{μ} hybridizes to S_{γ} very weakly—about two to four orders of magnitude more weakly than to S_{ϵ} or S_{α} . Among the γ genes, $S_{\gamma 3}$ hybridizes about 20–50 times more strongly with S_{μ} than do the other S_{γ} regions. Similar observations were made on mouse C_H genes¹⁸, and the relative sequence divergence of

S_{γ} raises questions about the H-chain switch mechanism. It has been suggested that sequences other than the S region repeats may be involved in switching³³, and it has also been proposed, on the basis of S-region homologies in the mouse, that γ genes are expressed initially by a switch from μ to the γ gene closest to μ , which can be followed by a switch from this γ gene to the other γ genes³⁴. In support of this latter proposal, we find that in humans, as in mice, the γ gene which seems to be closest to μ (that is, the γ_3 gene, see below) has the S_{γ} region which hybridizes most strongly to S_{μ} .

Order of the cosmid groups within the C_H locus

The cosmid clones described here fall into two groups which do not appear by their restriction maps to overlap either with each other or with the region around the μ and δ genes cloned in phage λ (refs 19, 27). However, we can predict the order of the two groups relative to each other and relative to the $J_H/\mu/\delta$ region by considering known deletions in an IgE-producing myeloma and two individuals with antibody deficiencies which are described in an accompanying paper³⁵. It has previously been shown by Southern blotting that the IgE-producing myeloma 266B1 has undergone a deletion of $\psi\epsilon_1$ (refs 25, 26). During the H-chain class switch all C_H genes between J_H and the newly expressed C_H gene are deleted, so assuming that the deletion in myeloma 266B1 results from H-chain switching, $\psi\epsilon_1$ must lie between the J_H region and the active ϵ gene. Therefore, region A (containing $\psi\epsilon_1$) apparently lies between the $J_H/\mu/\delta$ region and region B (containing ϵ). Further support for this gene order comes from our accompanying paper, where we show that individuals lacking α_1 antibodies and certain γ subclasses have a DNA deletion involving $\psi\epsilon_1$, the presumptive α_1 gene and three γ genes (apparently γ_1 , γ_2 and γ_4)³⁵. These data can be explained by a large inherited deletion which suggests the same order of the C_H genes as the deletion in the IgE-producing myeloma does. The overall gene order would therefore seem to be 5'- J_H -8 kb- μ -5 kb- δ ----- γ_3 -26 kb- γ_1 -19 kb- $\psi\epsilon_1$ -13 kb- α_1 ----- γ_2 -18 kb- γ_4 -23 kb- ϵ -10 kb- α_2 -3'. With the blocks of genes arranged in this way, all the genes have the same transcription orientation. The cosmid clones described here did not contain the two other known human C_H pseudogenes, $\psi\epsilon_2$ and $\psi\gamma$, so we cannot comment on the location of those genes.

Evolution of the C_H gene locus

We have shown here that the C_H gene organization of man is strikingly different from that of the mouse in that human γ , ϵ and α genes are interspersed with each other, whereas in the mouse the four γ genes are clustered upstream of the single ϵ and α genes. This arrangement in man probably results from duplication of a large chromosomal region containing γ , ϵ and α genes. Two models for the evolution of the human C_H locus

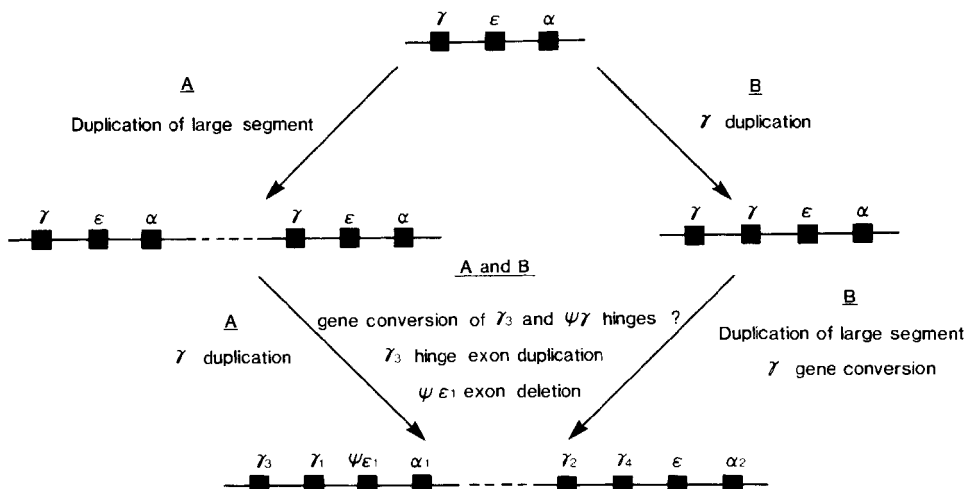


Fig. 5 Two models for the evolution of the human immunoglobulin C_H gene locus. The position of the $\psi\gamma$ gene is not known. $\psi\epsilon_2$ is apparently on a separate chromosome²⁴.

are illustrated in Fig. 5. The large ancestral segment which was duplicated may have had a gene order of either γ - γ - ϵ - α or γ - ϵ - α . In either case it is significant that when the protein sequences³⁶, gene sequences²² and flanking restriction site patterns (Fig. 1) of the four human γ genes are compared, it is clear that $\gamma 3$ and $\gamma 1$ are particularly closely related to each other, as are $\gamma 2$ and $\gamma 4$. If a segment with the gene order γ - γ - ϵ - α duplicated (Fig. 5B), then the γ gene homologies suggest the operation of gene correction mechanisms such as gene conversion which have kept adjacent γ genes similar. Alternatively, the homologies can be explained by initial duplication of a segment with the gene order γ - ϵ - α , (Fig. 5A), followed by further duplications of the γ genes. $\gamma 2$ and $\gamma 4$ could have arisen by a simple gene duplication. The production of $\gamma 3$, $\gamma 1$ and $\psi\gamma$ is evidently more complex, because $\gamma 3$, unlike the other γ genes, has four hinge exons, the most 5' of which is closely related to the single $\psi\gamma$ hinge exon while the other three are closely related to the single $\gamma 1$ hinge exon^{22,23}. It has been proposed that $\gamma 3$ was created by an unequal cross-over between $\gamma 1$ and $\psi\gamma$ ancestors, thus implying a gene order 5'- $\gamma 1$ - $\gamma 3$ - $\psi\gamma$ -3' (ref. 22). Our results do not seem compatible with this idea but suggest that exon duplication led to four $\gamma 3$

hinges originally all of the $\gamma 1$ type, followed by a gene conversion between the $\psi\gamma$ hinge and the first hinge of $\gamma 3$.

Several mechanisms have evidently contributed to the evolutionary diversification of the human immunoglobulin C_H gene cluster, including exon deletion in $\psi\epsilon 1$, exon duplication in $\gamma 3$, possibly gene conversion between γ genes, duplication of individual γ genes and duplication of a large chromosomal segment containing three different types of gene—the γ , ϵ and α genes. An interesting possibility is the involvement of S segments or their precursors in certain evolutionary changes in this region such as the duplication of C_H genes. It may be possible to derive a more precise description of the events in the evolution of the C_H gene locus from a study of the DNA of other primates. In any case, the structure of the human C_H gene locus, which has emerged to be substantially different from that of the mouse, clearly reveals a high degree of evolutionary flexibility in the development of this multigene family.

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The roles of individual polyoma virus early proteins in oncogenic transformation

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The expression in normal rat cells of modified polyoma virus genomes, separately encoding large T, middle T or small T antigens, has allowed the investigation of the roles of these proteins in oncogenic transformation. Middle T is sufficient to transform cells of established lines but the transformants are serum dependent. Large T lacks intrinsic oncogenic potential but can relieve the serum dependence of normal and transformed cells. Middle T alone cannot transform primary rat embryo fibroblasts.

GENETIC analysis of the respective roles in oncogenic transformation of the three known polyoma virus early proteins, large T, middle T and small T^{1–4}, is complicated by the overlapping organization of the coding sequences in the viral DNA^{5–7} (Fig. 1). The superimposed information content of the genomic DNA, contained in alternate translational reading frames, is resolved *in vivo* by differential processing of a common primary early region transcript: single introns of different lengths and

positions are excised to produce three distinct mRNAs which individually encode the proteins. Application of the contemporary gene transfer techniques to study the biological functions of the viral early proteins would be facilitated by prior resolution of the coding information at the DNA level. This was achieved initially for the middle T protein⁸ and more recently for the large T and small T proteins^{9,10} by precise deletion of intron sequences from otherwise full-length viral DNA cloned in a plasmid vector. Introns were removed by replacing genomic restriction fragments spanning them with the corresponding shorter fragments derived from cDNA clones¹¹. The three

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Table 1 Structure and coding capabilities of the recombinant plasmids

Plasmid	Polyoma DNA insert	Deletion (nucleotide)*	Coding capability		
			Large T	Middle T	Small T
pPY1	<i>Bam</i> HI genomic	None	+	+	+
pPyLT1	<i>Bam</i> HI genomic	Large T intron (410–794)	+	–	–
pPyMT1	<i>Bam</i> HI genomic	Middle T intron (747–808)	–	+	–
pPyST1	<i>Bam</i> HI genomic	Small T intron (747–794)	–	–	+
pNG1	<i>Bam</i> HI genomic	NG18 [†] (512–698)	+	–§	–§
pMC1	<i>Bam</i> HI– <i>Eco</i> RI	NG18 [†] (512–698)	Truncated amino terminal‡	–§	–§
pLT214	<i>Bam</i> HI– <i>Eco</i> RI	Large T intron (410–794)	Truncated amino terminal‡	–	–

* Nucleotide numbers in the polyoma DNA sequence according to ref. 37. † See refs 23–26. ‡ See text.

§ The common N-terminal polypeptide expected from translation in the middle T/small T frame up to the out-of-phase deletion (~10,000 daltons) has not been unambiguously identified in cells infected with the NG18 mutant, although mRNAs with the appropriate splices are synthesized (our unpublished results and G. Carmichael and T. Benjamin, personal communication).

recombinants generated (Fig. 1, Table 1) were designated pPyLT1, pPyMT1 and pPyST1 to indicate the intron deleted. Separate transfer of the modified genomes into cultured cells results in the production of the expected single early protein (refs 8, 9 and Z. Zhu, G. M. Veldman, A. C., A. C. and R. K., manuscript in preparation) through the efficient expression of unspliced mRNAs.

Many studies on polyoma virus-induced transformation^{12–20} have pointed to a critical role for the middle T protein, without excluding an involvement of all or parts of the other two early gene products. Experiments done with temperature-sensitive mutants of the large T protein have indeed suggested a role for the amino-terminal region of this protein in the maintenance of the transformed state in cell lines derived from FR3T3 rat cells²¹. The first set of results obtained with the modified viral genomes encoding only one of the early proteins showed that DNA of the middle T plasmid pPyMT1 transformed rat cells of the F2408 established line only slightly less efficiently than did cloned wild-type DNA. Transformed lines were obtained which synthesized the middle T, but neither the large T nor the small T protein. They grew in agar and to high density on plastic when tested in medium supplemented with 5% fetal calf serum. The 'middle-T-only' lines assayed for

oncogenicity all induced tumours in young Fischer rats in a manner indistinguishable from that of control lines producing all three early proteins. Although it was therefore clear for the first time that middle T expression alone is sufficient to initiate and to maintain an oncogenic state, significant alterations in cell physiology caused by the expression of large T and/or small T were still not excluded. It was suggested⁸ that if middle-T-only transformants could be shown to differ from other polyoma virus-transformed cells in some selectable property, it might be possible to assay for functions of the other viral proteins by transfer into the cells of appropriately modified genomes. We report here that such a property does exist. Cells transformed by plasmid pPyMT1 have a conditional defect in the expression of the transformed state: they revert to an apparently normal phenotype when grown in low serum medium. We show that the ability to maintain transformation at low serum concentration, which is characteristic of cells transformed by wild-type viral DNA, is specifically complemented by modified viral genomes that encode either all of the large T protein or only its N-terminal 40%. The same DNAs, while lacking transformation potential, could be used to derive FR3T3 lines with substantially reduced serum dependence. These results motivated us to ask whether the requirements for transformation of primary embryo fibroblasts differ from those of established cell lines. Successful transformation of these cells was achieved with cloned wild-type DNA, but not with any of the modified genomes separately encoding the early proteins.

Transformation of FR3T3 cells by DNA encoding only the middle T protein

The transforming activities of recombinant plasmids (Table 1) containing genes uniquely encoding large T (pPyLT1), middle T (pPyMT1) or small T (pPyST1) were tested with a Fischer rat cell line of the 3T3 type²², FR3T3²³. A recombinant containing unmodified viral DNA (pPY1) served as the control. DNA was transferred into cells by fusion with bacterial protoplasts²⁴. Frequencies of transformation were measured by focus formation in medium containing 10% calf serum (Table 2). As expected from previous results with *hrt* mutants^{25–27} (which express only the large T protein), plasmid pPyLT1 did not transform. Plasmid pPyMT1 transformed FR3T3 cells with a somewhat lower efficiency than the control plasmid pPY1, a result similar to that previously reported for F2408 cells⁸. Plasmid pPyST1, which encodes only the small T protein, did not transform. Additional plasmids were also negative: pNG1²¹, including the entire genome of the partial large T intron deletion mutant NG18 that expresses only the large T protein^{25–27}; pLT214, including a fragment of the viral DNA insert from pPyLT1 spanning the 5' half of the early region and encoding an N-terminal truncated form of the large T protein (334 virus-coded amino-acid residues plus 16 C-terminal residues from vector sequences); pMC1²¹, including the corresponding fragment from pNG1 and also encoding a truncated large T protein. Data

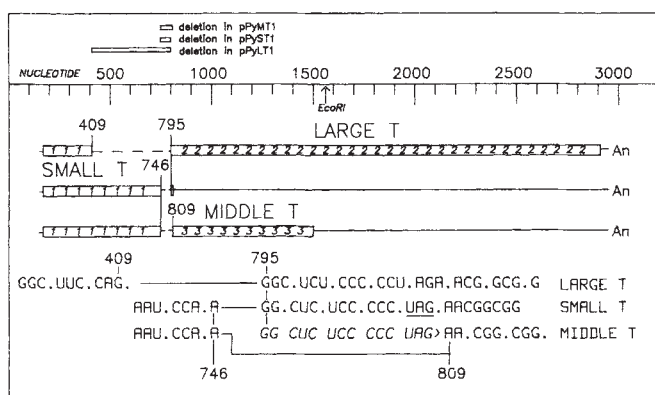


Fig. 1 Expression of the polyoma virus early region. A linearized map of the early region DNA is shown, marked with conventional nucleotide numbers³⁷ and the reference cutting site for endonuclease *Eco*RI. The three different spliced mRNAs are aligned with the map. Coding regions are boxed, non-translated regions are represented by continuous lines and introns by dashed lines. Numbers within the coding regions indicate the translational reading frame used; note that the 3' exon of the small T protein mRNA is translated in frame 1. RNA sequences across the splice junctions are shown in the lower part of the figure to illustrate how the joining of the two 5'-splice sites with two 3'-splice sites in three of the four possible combinations accomplishes the changes in codon reading frame required to use the two open frames available between nucleotides 809 and 1,497. The positions of the specific intron deletions in the viral genomes of plasmids pPyMT1, pPyLT1 and pPyST1 (see Table 1) are illustrated at the top of the figure.

shown in Table 2 for primary rat embryo fibroblast cultures will be discussed below.

Serum-dependent growth properties of transformants expressing only middle T protein

As illustrated in Fig. 2, FR3T3 cells grow to the monolayer stage in medium supplemented with 10% calf serum (a), but are unable to divide in only 0.5% serum (b) and die in growth medium without serum (c). Transformation with wild-type polyoma virus DNA, represented in Fig. 2 by the PYT21 line, derived by transfer of plasmid pPY1, enables the cells to grow past the monolayer stage to high density in either 10% (a) or 0.5% (b) serum, and also to grow relatively well without exogenous serum factors (c). By contrast, all lines derived from FR3T3 by transfer of pPyMT1 ('MTT' lines) grew to high density in 10% serum (a), but stopped at confluence in 0.5% serum (b). Even with frequent medium changes, these cultures could be kept at the monolayer stage for at least 2 weeks: neither cell death nor cell division could be detected by microscopic examination. The cells became larger and expanded on the plastic surface, a morphology reminiscent of that of the normal cells and different from that of wild-type polyoma virus transformants and of the same MTT cells in high serum medium. On addition of 10% calf serum, these cultures resumed their growth up to the high cell densities characteristic of transformed lines (data not shown). When serum was completely omitted, wild-type transformants could still grow, but MTT cells either grew very poorly or died over a period of 5–7 days (Fig. 2c). Below a critical serum concentration, cells which express only middle T thus have the growth properties on plastic of non-transformed cells. This also applies to their ability to divide in the absence of anchorage: all MTT lines tested so far exhibited a high cloning efficiency in agarose medium containing 10% serum (see also ref. 8), but, unlike cell lines transformed with wild-type viral DNA, none of them could form even microcolonies when the serum concentration was reduced to 0.5% (Table 3). This differential serum requirement of cells expressing only middle T has been confirmed without exception using other FR3T3-derived MTT lines, as well as middle-T-only lines derived from F2408 (see Table 3, ref. 28 and P. Kaplan and B. Ozanne, personal communication).

Table 2 Efficiency of transformation of FR3T3 rat cells and of rat embryo fibroblasts by plasmids encoding individual polyoma virus early proteins

Plasmid	FR3T3 cells	Foci per plate	
		Rat embryo fibroblasts	
		Expt 1	Expt 2
pBR322	0	0	0
pPY1	128	56	256
pPyLT1	0	0	NT*
pNG1	0	0	NT
pPyMT1	90	0	0
pPyST1	0	0	NT
pMC1	0	NT	NT
pLT214	0	0	NT

Confluent primary cultures, prepared as described in ref. 38 from 14-day-old Fischer rat embryos and parallel cultures of an early passage of the FR3T3 line²¹ were seeded at a density of 2×10^5 cells per 35-mm Petri plate (Lux) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (Gibco). After 24 h, the cells were fused with bacterial protoplasts (5×10^3 to 10^4 protoplasts per cell) of *E. coli* 1106 bacteria carrying the indicated plasmids, according to our previously published procedure²². Three days later, cells were trypsinized and plated at a density of 10^5 cells per 35-mm Petri plate. Cultures were stained with Giemsa and foci were counted after 10 days for FR3T3 cultures and after 1 month for rat embryo fibroblast cultures (no further increase in the number of foci was observed after 8 weeks incubation of parallel plates for both types of cultures). Numbers are total foci per 8×10^5 FR3T3 cells, 4×10^5 rat embryo cells in expt 1 and 2×10^6 in expt 2. NT, not tested.

Table 3 Growth in agarose medium of normal, wild-type polyoma-transformed and pPyMT1-transformed rat lines

Cell line	Colony-forming ability in agarose medium supplemented with:	
	10% Calf serum	0.5% Calf serum
FR3T3	<0.001	<0.001
PYT21	100	14
4.2	100	20
MTT1	100	<0.001
MTT4	100	<0.001
2.9	100	<0.001

10^5 cells of the indicated line were seeded in agarose medium supplemented with the indicated amount of serum. Microcolonies per microscope field were counted after either 8 days (10% serum) or 15 days (0.5% serum) and values were normalized as per cent of the cell input. The origin of cell lines MTT1, MTT4 and PYT21 is indicated in Fig. 2 legend. The same results were obtained for three other MTT lines independently isolated from FR3T3 (MTT2, MTT3, MTT5). Transformed lines 4.2 and 2.9 were derived⁸ from F2408 Fischer rat established fibroblasts after transfection with plasmids containing either wild-type (4.2) or pPyMT1 polyoma (2.9) DNA.

Can serum-dependent MTT transformants be complemented by other viral proteins?

To assess whether the serum-dependent transformed phenotype of MTT transformants could be complemented by one of the other early viral proteins, MTT4 cells were fused with bacterial protoplasts carrying various plasmids, seeded in medium containing 0.5% serum and submitted to selection for stable transformants (Table 4). Colonies in agarose medium were observed, not only after transfer of a complete transforming region, but also with plasmids pNG1, pLT1, pMC1 and pLT214, that encode either a full-size or a truncated large T protein and cannot by themselves transform FR3T3 cells (see Tables 2, 5). Similar complementation results were obtained when focus formation at low serum concentration was used as the transformation assay (data not shown).

Several agar colonies and foci resulting from complementation were picked and grown into cell lines at low serum concentration. They all now had the growth properties characteristic of wild-type transformants. Representative cell lines, complemented with the pPyLT1 plasmid, were tested for the expression of the large T protein: unlike the parental MTT4 cells, they exhibited the nuclear staining by immunofluorescence with anti-T antigen serum characteristic of the large T protein (data not shown) and immunoprecipitation of T antigens demonstrated the expression of a full-size large T protein (Fig. 3a). A novel immunoprecipitable polypeptide could be detected in MTT cells complemented by DNA encoding only the N-terminal 40% of the large T polypeptide (plasmid pMC1, see Table 1) (Fig. 3b). Like the full-size large T protein, it could be labelled *in vivo* with ^{32}P -orthophosphate. Its apparent molecular weight (40,000) closely corresponds to the size predicted from the DNA sequence (36,700) and is identical to that of an immunoprecipitable polypeptide previously identified in a cell line (FR3T3-BR70, see below) isolated after transfer of plasmid pMC1 in FR3T3 cells²¹. Although further characterization of this protein is required, these results support the genetic evidence that the activity of a truncated large T protein can complement the serum dependence of MTT transformants.

No efficient complementation was observed in the progeny of MTT cells fused with pPyST1 protoplasts (Table 4). However, prolonged incubation of these plates produced a significant number of very small colonies in agarose medium, or foci on plastic plates. A representative number of these colonies were picked, but we have been unable to establish from these cell lines able to grow in low serum medium.

Selection of transformants at low serum concentration

The results described above suggested that pPyMT1 DNA alone was unable to transform FR3T3 cells if transformation were

monitored by growth at low serum concentration²⁹. We would further expect transformation in such conditions to be complementable by plasmids able to rescue the serum factor dependence of MTT transformants. In the experiment reported in Table 5, FR3T3 cells were fused to pairs of protoplasts carrying either pBR322 or pPyMT1 plus other viral recombinants or pBR322. Transformed colonies were quantified in agarose medium containing either 10 or 0.5% serum, and focus formation was measured in liquid medium containing 0.5% serum. The results confirmed both expectations. Transfer of pBR322 plus individual polyoma recombinants yielded transformation in 10% serum with genomic (pPY1) or middle T (pPyMT1) plasmids, but in 0.5% serum, only with the pPY1 genomic plasmid. Complementation of pPyMT1 transformation in 0.5% serum was clearly observed with the recombinant genomes that encode at least the amino-terminal 40% of the large T polypeptide (pPyLT1, pNG1, pMC1). Transfer of pPyMT1 plus the plasmid that encodes only the small T protein (pPyST1) did not yield visible foci in low serum medium, except after prolonged incubation (4 weeks) of the plates, where several very small foci were found. This effect of pPyST1, similar to that observed in the complementation of MTT cells (see above), also requires further study.

Selection for growth at low serum concentration in the absence of the middle T protein

Our complementation data showed that the characteristic very low requirements for serum growth factors of polyoma virus-transformed cells is under the genetic control of the large T gene. To test whether this phenotype can be acquired without expression of the middle T protein, selection for growth at low serum concentration was applied after transfer of the plasmids that code for the amino-terminal end of large T into cells of the highly serum-dependent FR3T3 line (see Fig. 2). FR3T3 cells, after transfer of vector DNA alone (Table 6), had a very low plating efficiency ($\sim 2 \times 10^{-3}$) in medium supplemented with 0.5% serum. This was increased by 5–10-fold (Table 6) when the cells had been fused with protoplasts carrying either pPyLT1 (or pNG1) or pLT214 (or pMC1). These clones could be further grown in low serum conditions into cell lines. In the presence of 10% serum, these lines had growth characteristics similar to those of the parental FR3T3 cells: they retained a low saturation density (Table 6) and did not grow in suspension. They could, however, be differentiated from the parental cells

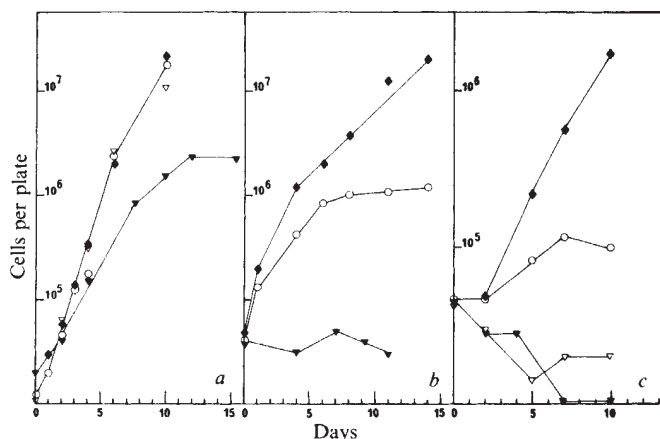


Fig. 2 Growth of normal FR3T3 cells and representative MTT and wild-type polyoma transformants at various serum concentrations. Cells were seeded at a density of 5×10^4 cells per 60-mm Petri plate in DMEM supplemented with either 10% (a) or 0.5% (b) serum or without addition of serum (c). The medium was changed every other day below 10^6 cells per plate and every day above this value. At the indicated times, plates were trypsinized and counted. MTT1 and MTT4 lines were derived from FR3T3 cells by focus formation in medium containing 10% serum after transfer of plasmid pPyMT1. PYT21 was established in a parallel experiment after transfer of plasmid pPY1. $\blacktriangledown$: FR3T3; $\blacklozenge$: PYT21; ∇ : MTT1; $\circ$: MTT4.

Table 4 Complementation of MTT cells for growth in agarose medium at low serum concentration

Fusion of MTT4 cells with protoplasts carrying	Colonies per 10^5 cells	
	17 days	7 weeks
pBR322	0	0
pPY1	30	47
pPyMT1	0	0
pNG1	20	38
pPyLT1	8	32
pMC1	12	20
pPyST1	0	15*
Control without fusion	0	0

MTT4 cells were seeded (2×10^5 cells per 35-mm plate) in DMEM containing 0.5% serum. They were fused the next day with 10^4 protoplasts per cell of *E. coli* 1106 bacteria carrying the indicated plasmids. After 24 h, cells were trypsinized and seeded (10^5 cells per 60-mm plate) in agarose-gellified DMEM containing 0.5% newborn calf serum. Colonies were first counted after 17 days and again after 7 weeks. Numbers are averages of six plates.

* Minute colonies of slow-growing cells.

by their ability to grow to a low saturation density in the presence of 0.5% serum; curiously, the growth of such lines in low serum medium was similar to that of MTT lines in the same conditions (Table 6). Immunofluorescence measurements on representative lines derived after transfer of pPyLT1 showed positive nuclei characteristic of the expression of the large T protein. This is consistent with the conclusion that, at least after transfer of the entire large T gene, the modification in serum requirement results from the expression of a large T related polypeptide.

In an independent experiment²¹, plasmid pMC1 (see Table 1) was transferred into FR3T3 cells and progeny colonies, grown in 10% serum, were randomly screened for the expression of a viral nuclear T antigen. Out of the 77 clones tested, 6 were weakly positive and one of them, FR3T3-BR70, was kept for further analysis. These cells were shown²¹ to synthesize a protein immunoprecipitable with anti-polyoma virus T antigen, with the expected molecular weight of 40,000, identical to that of the antigenic protein now observed in MTT cells complemented by pMC1 plasmid (see above Fig. 3). The cells had the same morphology, growth inhibition in suspension and low saturation density (Table 6) as the original FR3T3 cells when studied in the presence of 10% serum. However, the results described above would imply that, unlike FR3T3, they should grow in medium supplemented with 0.5% serum. This prediction is confirmed by the data shown in Table 6, strongly suggesting that expression of the N-terminal portion of the large T protein is sufficient to reduce the growth factor requirement of FR3T3 cells.

DNA's separately encoding the early proteins do not transform primary embryo fibroblasts

We have thus far described experiments with FR3T3 rat cells which demonstrate an effect of the large T gene on cellular growth regulation: expression of the large T protein, or only its N-terminal region, confers on both parental and middle T-transformed FR3T3 cells a reduced dependence on serum growth factors. To test the more general significance of our findings, we decided to investigate the requirements for transformation of non-clonal fibroblast cultures derived from primary explants of rat embryos ('primary' cells). Polyoma virus is known to be able to transform primary rodent cells. In addition to the transformation characters observed in transformed derivatives from established normal cell lines, it was recognized that virus transformation enables the cells to grow in culture for an unlimited period³⁰. When plasmids containing wild-type or modified viral genomes were transferred into primary rat fibroblasts, transformation resulted only with the wild-type DNA (Table 2). Plasmid pPyMT1, encoding only the middle T protein and active in parallel experiments in the transformation of FR3T3 cells, did not transform primary cells,

even in medium containing 10% serum. Experiments are in progress to determine whether the pairwise transfer of early genes would transform these cells.

Concluding remarks

We have confirmed previous evidence that the middle T protein alone can confer on established cell lines a phenotype similar to that of wild-type polyoma-transformed cells. Expression of this protein, however, was not sufficient to maintain the transformed state in low serum growth conditions in the highly serum factor-dependent line FR3T3. The low serum growth defect of FR3T3 lines expressing only the middle T protein could be complemented with equivalent efficiencies by DNA encoding either all or no more than the N-terminal 40% (334 amino acids) of the large T protein. Similarly, co-transfer of DNA encoding either the full-size large T protein or only its N-terminal region complemented the ability of the middle T gene to transform FR3T3 cells in low serum medium, yielding transformants which were not dependent on exogenous growth factors for expression of the transformed phenotype. Transfer of the full-size or truncated large T gene alone was sufficient to promote growth of normal FR3T3 cells at low serum concentrations, but did not induce expression of other properties (lack of topoinhibition, anchorage independence) characteristic of transformation. We therefore conclude that the middle T and large T gene products have independent effects on cellular physiology which together are responsible for the phenotype of wild-type transformants.

An important motivation for the collaborative efforts reported here was to resolve the apparent discrepancy between the

Table 5 Transformation frequencies after co-transfer into FR3T3 cells of polyoma sequences encoding different early proteins

Bacterial protoplasts carrying:	Transformants per 10 ⁵ cells in DMEM supplemented with:		
	10% Calf serum	Agar	0.5% Calf serum
	Foci		Foci
pBR322	0	0	0
pBR322 + pPY1	95	48	65
pBR322 + pNG1	0	0	0
pBR322 + pPyLT1	0	0	0
pBR322 + pMC1	0	0	0
pBR322 + pPyMT1	74	37	0
pBR322 + pPyST1	0	0	0
pPYMT1 + pNG1	79	16	31
pPYMT1 + pPyLT1	NT	15	39
pPYMT1 + pMC1	46	14	22
pPYMT1 + pPyST1	60	27	0*

FR3T3 cells (2×10^5 cells per 35-mm plate) were fused either with 2×10^9 protoplasts of *E. coli* 1106 carrying pBR322 or with the same total number of protoplasts prepared from mixed cultures (50:50) carrying the indicated plasmids. After 24 h, two plates from each group were trypsinized and the cells were seeded in four plates, two in liquid medium and two in agarose medium, in DMEM supplemented with 10% calf serum. Four days later, two more plates from each group were transferred into two 10-cm plates in medium supplemented with 0.5% serum. Foci and agar colonies were counted at day 16. Numbers are means of two plates (10% serum) or four plates (0.5% serum).

* Minute foci of slow-growing cells were observed after prolonged incubation of the plates (4 weeks).

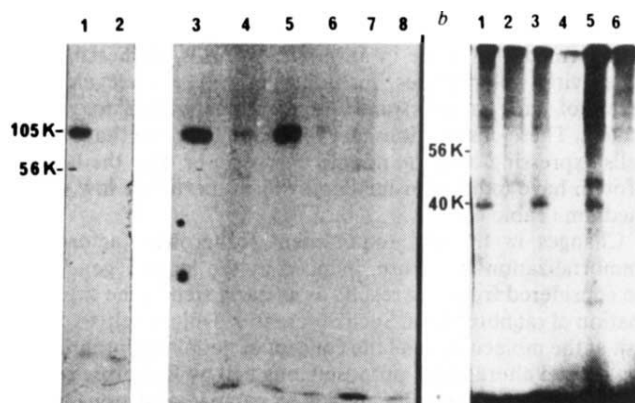


Fig. 3 Immunoprecipitation analysis of the viral proteins synthesized in cells derived from MTT transformants by complementation with the full-size or truncated large T genes. Cell lines MLT1 and MLT2 were independently derived from MTT4 cells after transfer of plasmid pPyLT1, and cell lines MMC1, MMC2 and MMC3, from MTT4 cells after transfer of plasmid pMC1 (see Table 1), by selection for colonies grown in agarose medium in the presence of 0.5% serum. Cells were subcloned twice in succession and routinely maintained in DMEM containing 0.5% serum. Before labelling, the cells were seeded at a density of 10^5 cells per 10-cm Petri plate and grown for 4 days in the presence of 10% serum. Labelling was for 2 h in the presence of $250 \mu\text{Ci}$ of ^{32}P -orthophosphate (Amersham France) in 1 ml of Tris-buffered isotonic saline solution. Extracts were prepared and analysed by immunoprecipitation with anti-polyoma T antigen immune serum as described in ref. 21. Concentration of polyacrylamide was 10% for the gel shown in *a*, and 12.5% for that in *b*. The indicated electrophoretic mobilities corresponding to apparent molecular weights of 105,000, 56,000 and 40,000 were deduced from the position of protein markers on the same gels²¹. *a*: lane 1, MLT2 cells, immune serum; lane 2, MLT2 cells, control serum; lane 3, MLT1 cells, immune serum; lane 4, MLT1 cells, control serum; lane 5, PYT21 cells, immune serum; lane 6, PYT21 cells, control serum; lane 7, MTT4 cells, immune serum; lane 8, MTT4 cells, control serum. *b*: lane 1, MCC3 cells, immune serum; lane 2, MCC3 cells, control serum; lane 3, MCC2 cells, immune serum; lane 4, MCC2 cells, control serum; lane 5, MCC1 cells, immune serum; lane 6, MCC1 cells, control serum.

observation that middle T expression alone was sufficient to transform established rat cells of the F2408 line⁸ and reports that at least the N-terminal region of the large T gene was additionally essential to maintain the transformed state in lines derived from FR3T3 by infection with the *tsa* mutant of polyoma virus²¹. We found that cell lines derived from FR3T3 after transfer of the pPyMT1 plasmid conditionally express the transformed phenotype in a manner dependent on growth factor concentration. The same DNAs which were able to complement *tsa* transformants for transformation at the restrictive temperature were shown to complement the serum-dependent defect of cell lines expressing only middle T. Thus, the present experiments confirm the original suggestion³¹ that the large T protein, or only its N-terminal region, plays an important part in the maintenance of transformation.

We still, however, cannot explain why FR3T3 cells after transfer of the middle T gene have only a serum-dependent conditional defect in transformation, whereas *tsa* virus transformed cells of the N type revert to a normal phenotype at 40 °C in the presence of 10% serum³¹. We recently transferred into MTT FR3T3 cells a viral genome encoding only large T and carrying the *tsa* mutation (M.R. and F.C., unpublished experiments). These cells display a serum-dependent transformed phenotype at 40 °C but not at 33 °C. This result provides further important evidence for the continuous requirement for a large T gene product to maintain transformation in low serum medium, but differs from that obtained with *tsa*-transformed cells. The difference may result from the establishment of the transformed lines in recent experiments by DNA-mediated transfer of a large number of gene copies per cell, rather than by virus infection at low input multiplicities³¹ in the former study. Also, the *tsa* transformants were established at the permissive temperature, in the presence of an active large T protein. A role for this protein in the formation of the head-to-tail tandemly integrated viral genomes commonly observed in virus transformants was indicated by recent experiments using *tsa* mutants³². Another approach is suggested by results obtained with temperature-dependent SV40 *tsa* transformants³³ indicating that these cells behave as transformed cells at 40 °C in the presence of 20% serum, but not with only 10%

Table 6 Derivation of cell lines with reduced serum requirement by selection in medium containing 0.5% calf serum after transfer into FR3T3 cells of large T coding genes

a, Plating efficiency of FR3T3 cells after transfer of plasmids encoding large T

Plasmid	No. of colonies	
	5×10^3 Cells per plate	10^4 Cells per plate
pBR322	10	28
pPyLT1	80	150
pNG1	100	NT
pLT214	60	132
pMC1	89	160

b, Growth of FR3T3 cells and of FR3T3 derivatives selected after transfer of genes encoding large T

Cell line	Relative cell growth in DMEM containing:		
	10% Serum (day 8)	0.5% Serum (day 8)	0.5% Serum (day 15)
FR3T3	60	1	1
PYT21	800	70	400
FR3T3-BR70	110	9	41
FR3T3-LT1	100	12	38
MTT4	800	14	40

a, FR3T3 cells were fused with protoplasts carrying the indicated plasmids as described in Table 2 legend. After 24 h, the cells were trypsinized and replated at the indicated densities in 10-cm Petri plates in medium supplemented with 0.5% serum. Giemsa staining and colony counting were done 1 month later. In these conditions, the efficiency of cloning of the FR3T3 cell line in DMEM containing 10% newborn calf serum is of the order of 50%. b, FR3T3-BR70 cell line was derived from FR3T3 cells after transfer of plasmid pMC1 (ref. 19, and see text) and FR3T3-LT1, after transfer of plasmid pPyLT1 by selection of clones in medium containing 0.5% serum (a). Cells were seeded at day 0 at a density of $1-5 \times 10^4$ cells per 60-mm Petri plate, depending on the line, in medium containing the indicated concentration of serum. Cells of parallel cultures were trypsinized and counted at days 0, 8 and 15. Values shown are ratios of cell number at the indicated time to that at day 0. Values underlined correspond to confluent arrested monolayers. These cultures resumed their growth after trypsinization and seeding at lower density in the same medium.

serum. Both polyoma *tsa* and MTT lines could thus in fact be serum-dependent transformants, the critical serum concentration being higher for the *tsa* lines than for MTT cells. Experiments are in progress to test these hypotheses.

Our present data suggest that the region of the large T protein involved in the alteration of cell growth control is localized within its amino-terminal 334 residues. This genetically defined domain would retain its biological activity after deletion of other parts of the polypeptide chain, as previously demonstrated for various multifunctional proteins, such as *Escherichia coli* DNA polymerase I³⁴. Experiments using available mutants³⁵

are in progress to localize more precisely this functional domain. It may be encoded by the segment of genomic DNA with two open reading frames that also encodes the C-terminal half of the middle T protein (see Fig. 1). Deletions within this region of the genome are already known to affect transformation³⁵. Are the two functions of polyoma virus which cause oncogenic transformation encoded by alternative reading frames in a single DNA sequence?

We further show here that DNA encoding only the middle T protein does not stably transform rat embryo fibroblasts. We suspect that transformation of primary cells also requires a function exerted by the large T protein, that would confer on cells an unlimited growth potential in culture ('immortalization' function). Recent experiments demonstrated that transfer of DNA encoding only large T into primary rat embryo cells allows the establishment of permanent cell lines which do not express other transformation characters; these lines, as one might predict, could be efficiently transformed by plasmid pPyMT1 DNA (ref. 10 and M.R., Z. Naghashfar, N.G., R.K. and F.C., manuscript in preparation). This function of the large T gene may be related to its ability to reduce the serum requirement of established cell lines because it is also retained by the genes encoding only the N-terminal part of the protein.

Responsiveness to growth factors, modulated by the large T protein, may thus appear as critical both in the expression of oncogenicity and in the acquisition by primary cells of an unlimited growth potential in culture. Further biochemical investigations should define the nature of the growth factors involved. Recent experiments on cell lines expressing only the middle T protein (ref. 28 and B. Ozanne, personal communication) indicate that these cells secrete in the culture medium growth factor(s) that transiently induce the expression of a transformed phenotype in normal cells. Cells synthesizing the other viral polypeptides, including large T, secrete elevated levels of such factor(s) and are more responsive to growth factors. These observations may account for the fact that FR3T3 cells expressing only the middle T protein or only the large T protein have indistinguishable growth properties in low serum medium (Table 6).

Changes in the cell requirement for growth factors and immortalization in culture, induced by the large T gene, may be considered from our results as an early step in the transformation of rat fibroblasts. Such observations might help to establish at the molecular level the concept of neoplastic progression by 'graded alterations', proposed long ago by Rous (see ref. 36 for review) and originally applied to papova virus transformation by Vogt and Dulbecco³⁰.

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Making ends meet: a model for RNA splicing in fungal mitochondria

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On the basis of available nucleotide sequence and genetic data, we present a model for RNA splicing in fungal mitochondria. Seven intron RNAs of two fungal species can form identical secondary structures, involving four conserved sequences, which bring the ends of each intron together and allow an internal guide RNA sequence to pair with exon bases adjacent to the splice junctions. The splicing sites are thus aligned precisely within a conserved structure, which we suggest could present specific recognition signals to the proteins that catalyse the splicing reaction.

MANY eukaryotic genes are interrupted by DNA sequences which do not code for any part of the gene product. These DNA sequences are known as introns (or intervening sequences), whereas the coding regions of the gene are called exons. Introns have been found in eukaryotic mRNA, tRNA and rRNA nuclear genes, and also in mitochondrial mRNA and rRNA genes of fungi¹⁻³ and at least one plant mitochondrial mRNA gene⁴. In all cases introns are transcribed as part of the primary RNA transcript and are then excised by 'splicing'—a precise cleavage and ligation reaction which leaves the various exons joined together end to end in their order of synthesis. The mechanism of splicing has not been fully elucidated for any intron. We now present a model which shows how the RNA of one class of mitochondrial intron could take up a well defined secondary structure, allowing the precise and unambiguous alignment of the ends of exons flanking the intron ready for the cleavage and ligation reactions. We have used two lines of evidence in constructing the model. Comparison of the DNA sequences of four mitochondrial introns of *Aspergillus nidulans* which we recently determined (ref. 5 and R.B.W. *et al.*, in preparation) and five of *Saccharomyces cerevisiae*⁶⁻⁸ highlights certain key conserved sections of intron

sequence. When these are taken as the basis of the formation of base-paired regions of the RNA molecule, further conserved RNA-RNA pairing between non-conserved sequences is revealed, and quite remarkably all these introns can form very similar RNA secondary structures. Genetic evidence, provided largely by the work of Slonimski and co-workers⁹⁻¹², shows that particular short segments of the intron sequence distant from the splicing sites are important in *cis* for splicing; our model explains the phenotypes resulting from *cis*-acting mutations.

The introns studied are listed in Table 1 and the terminology used is described in Fig. 1 legend. The sequences that are highly conserved in introns of both species⁵ are named P, Q, R and S; their sequences and locations are given in Table 1. Seven of the introns have the internal primary structure shown in Fig. 1^{9,13}: a *cis*-acting region occupies the 5' end of the intron (which in mRNA introns has an open reading frame), a central protein-coding region extends from just beyond conserved sequence R to the stop codon and there is a 3' *cis*-acting region with no open reading frame. The protein-coding region of YC4 has been shown^{9,13} to code for a protein essential for the excision of YC4 ('mRNA maturase')¹¹, and this is probably the case for several other introns. The general features of the model will

Table 1 Important sequences within mitochondrial introns

Intron	5' Splice Site	IG	E	P	Q	R	E'	S	3' Splice Site
		*							
YC4	↑- 6-	AUGGCCU	-32- UGUGC	-3-AUGCUGGAAA-192-AAUCAGCAGG-65-UCAGAGACUACA-	1-GCACA	-1137-AAGAUUAGUCC-	21-↑		
YOX4	↑- 6-	GUGGCC	-34- UUUUC	-3-AUGCUGGAAA- 78-AAUCAGCAGG-50-UCAGAGACUACA-	1-GAAAAA	- 740-AAGAUUAGUCC-	36-↑		
Y ₆₀	↑- 3-UUACCCCUUGUCCC	-64-AUUGGGUG	-2-UUGCUUAGAU-	25-AAUAAGCAGC-49-UCAACGACUAGA-34-CGCCCAAU-848-AUGAUUAGUCU-	61-↑				
YOX3	↑-1005- UAAUAAGAUGCCG	-28- CAACAC	-3-AUCGGGAAA-165-AAUCGCGCAGG-95-UCAGAGACUACA-	1-GUGUUG	- 28-AAGAUUAGUCC-	29-↑			
NOX1	↑- 250-	AAGCCG	-27- CUUCGC	-3-AUGCAUGGAA-102-AAUAUGCAGG-39-UCAGAGACUACA-	1-GCGAGG	22-AAGACAUAGUCC-362-↑			
NOX2	↑- 9-AUAGAAAACUGCCGGC-	6- UUUGGC	-3-AUACUGGAAA- 28-AAUCAGUAGG-32-UCAGAGACUUA-	1-GCCAAA	869-AAAAUAGUCC-124-↑				
NOX3	↑- 1-AAAAAUUAAGGCUUCU-	3- CGUUGC	-3-AUGCUGGGAC- 69-UAUCAGCAGG-19-UCAGAGACUUUA-	1-GCAACG	- 851-AAGAUAAAGUCC-	83-↑			
NC	↑- 4-	ACAGAUAACC	-26- UCCUGC	-3-AUGCUGGAAA- 74-AAUCAGCAGG-30-UCAGAGACUACA-	1-GCAGGA	- 764-AUGAUUAGUCC-	76-↑		
YC3	↑- 2-	AUAUAGAGGAUCC	-						↑
Consensus				7878667586 AUGCUGGAAA	7886687887 AAUCAGCAGG	888778888758 UCAGAGACUACA		867878788887 AAGAUUAGUCC	

The nucleotide sequences and locations within nine fungal mitochondrial introns of regions that are important for RNA splicing, including the conserved sequences P, Q, R and S, are shown. The latter are named in their order of occurrence in the 5' to 3' direction within the introns. The E, E' and IG sequences are described in the text. Numbers indicate distances in bases between particular sequences. The asterisk marks the conserved G base of the IGS. P and Q are the same as A and B, respectively, in ref. 5.

be presented first, followed by a detailed consideration of the supporting evidence.

A model of mitochondrial RNA splicing

Figure 2 gives a diagrammatic representation of the steps in the splicing process, and Fig. 3 shows the actual secondary structure of the folded intron RNA. The model proposes three major stages in the excision of these introns.

(1) Intron RNA forms a specific secondary, and presumably tertiary, structure involving a number (usually nine) of conserved RNA-RNA interactions, with the result that the ends of the intron are brought close together. The RNA-RNA pairings involving the conserved sequences P, Q, R and S are among the most critical. The P and Q sequences are synthesized early, and their pairing P4 and the associated P5 (Fig. 3) are important in nucleating the secondary structure. The R-S pairing P7 and the associated P8 (Fig. 3) bring the 3' end of the intron close to R, which is in turn brought close to the 5' end of the intron by the pairing of the E and E' sequences. Proteins may also be involved, binding at specific sites on the intron RNA either to stabilize secondary structure or to aid the formation of the precise alignment structure in stage 2. We regard the characteristic secondary structure formed by pairings P3 to P9 in Fig. 3 as the 'core structure' of these introns. The formation of the core structure is a prerequisite for the next stage.

(2) An internal guide sequence (IGS) located just 5' to the E sequence (Figs 1, 3) pairs with short stretches of RNA sequence at the end of the upstream exon and the beginning of the downstream exon, acting as an adaptor molecule that brings the two splicing sites tightly together, separated only by the length of a phosphodiester bond. The IGS-upstream exon pairing is P1 in Fig. 3, the IGS-downstream exon pairing is P10. The P1 and P10 pairings involving IGS make up the

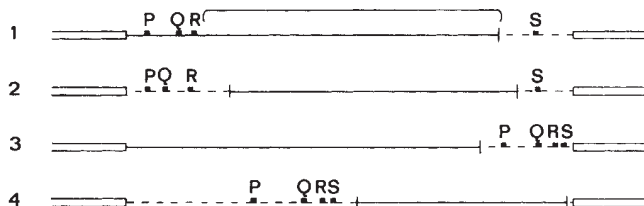


Fig. 1 A schematic representation of the internal primary structure of a class of fungal mitochondrial introns. Four different arrangements of open reading frames are shown; class 1 is the majority arrangement, found in six of the nine introns discussed here, while the other classes have one representative each. Internal features of each intron are shown to scale, but all introns have been scaled to the size of the class 1 intron. Symbols are as follows: open bars, exon sequences; continuous line, open reading frame within an intron; discontinuous line, intron sequence with no open reading frame. The bracketing line above the class 1 intron indicates the part of the yeast cob I4 intron in which trans-acting mutations of the box7 class have previously been located; this defines the maturase coding region. P, Q, R and S are the conserved sequences shown in Table 1. The results presented in this article are based on the comparison of nine intron DNA sequences, four from *A. nidulans* and five from yeast. We refer to these and other introns by species, gene and number only, symbolizing *S. cerevisiae* by Y and *A. nidulans* as N, and dropping 'intron'. Thus, yeast introns are written as follows: cob I3, YC3; cob I4, YC4; oxi3 I3, YOX3; oxi3 I4, YOX4; 21S rRNA_ω, Yω. *A. nidulans* introns are written as follows: cob A11, NC; oxi A11, NOX1; oxi A12, NOX2; oxi A13, NOX3. The data for Yω (ref. 8), YC4 (ref. 7), YOX3 and YOX4 (ref. 6) have been published, and those for NC, NOX1, NOX2 and NOX3 are from our laboratory and will be published in detail elsewhere (ref. 5 and R.B.W. *et al.*, in preparation). Nine introns NC, NOX1, NOX2, NOX3, YC3, YC4, YOX3, YOX4 and Yω, have been studied but we only show analyses based on eight of the introns plus those portions of the YC3 sequence that have already been published²¹. In this figure intron 1 = YC4 (YOX4, YC3, NC, NOX2 and NOX3 are similar to YC4), 2 = Yω, 3 = YOX3 and 4 = NOX1.

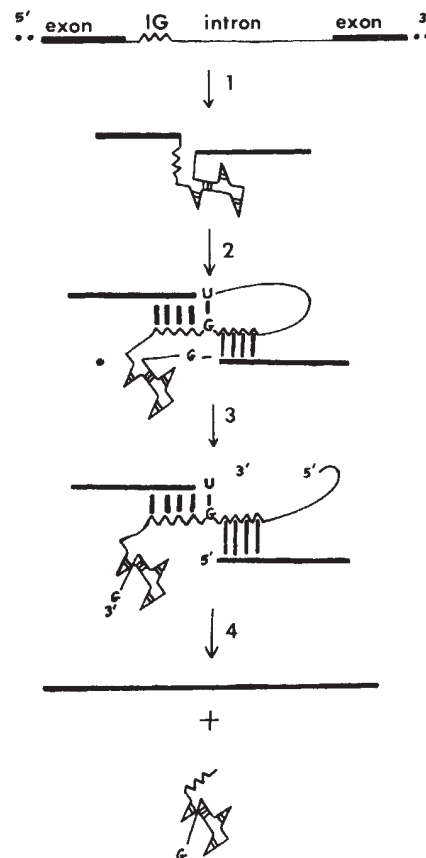


Fig. 2 Model of the role of the RNA substrate in mitochondrial RNA splicing. The model is described in the text. The steps shown here are: (1) formation of internal secondary structure; (2) formation of the precise alignment structure; (3) endonuclease binding followed by specific cuts to produce the ends shown and diffusion of free intron ends away from the splicing complex; (4) ligation of exon ends and release of linear intron RNA from the splicing complex. The precise alignment complex which is P1 + P10 of Fig. 3, is shown greatly enlarged between stages (2) and (4). The thick line represents exon RNA, the thin line intron RNA, the zigzag line the internal guide RNA sequence (IGS).

precise alignment structure, details of which are given in Fig. 6 for nine introns. In all cases the splice sites are precisely aligned, and are in similar chemical environments at the ends of symmetrically placed helices. As shown in Fig. 2, this structure also ensures that exon-exon ligation is favoured, because when the RNA is cut at the exon-intron junction the exon ends are held in the double-stranded region, while the intron ends are free to diffuse away.

(3) The completed precise alignment structure is recognized by the splicing protein(s). The features recognized are the RNA structure *per se*, and certain chemical groups that only occur together near the splice sites in the complete structure, in particular the U·G base pair (bp) of P1 adjacent to the 5' splice site and the G base at the 3' end of the intron. In addition, the protein(s) recognizing this structure may also need to bind to components of the core structure or its associated proteins so as to bind efficiently. The specificity of the reaction is dictated by other non-conserved chemical signals and by the mRNA maturase¹³. Endonucleolytic scission at the 5' and 3' splice sites may be simultaneous or sequential. The free ends of intron RNA diffuse away immediately but the free ends of exon RNA are still held in the P1 and P10 pairings, and are ligated together by an RNA ligase which need not be specific. The splicing proteins are released from the IGS; the P1 and P10 pairings fall apart, releasing the linear intron (which is presumably subsequently destroyed) and the spliced mRNA.

The core structure

The fact that all introns considered here possess the same core secondary structure argues for the functional importance of this structure. This is strongly supported by our ability to explain the phenotype of all the sequenced *cis*-acting mutations affecting splicing, and by the maintenance of intact base-pairing despite variations from the consensus sequences. We stress that if any one intron RNA sequence is considered alone it is possible to find alternative ways of pairing the conserved sequences, but the model secondary structure presented in Fig. 3 is applicable to all the introns of both species. We have only considered pairings to be potentially biologically meaningful if they occur between identical or identically placed sequences of all the introns studied.

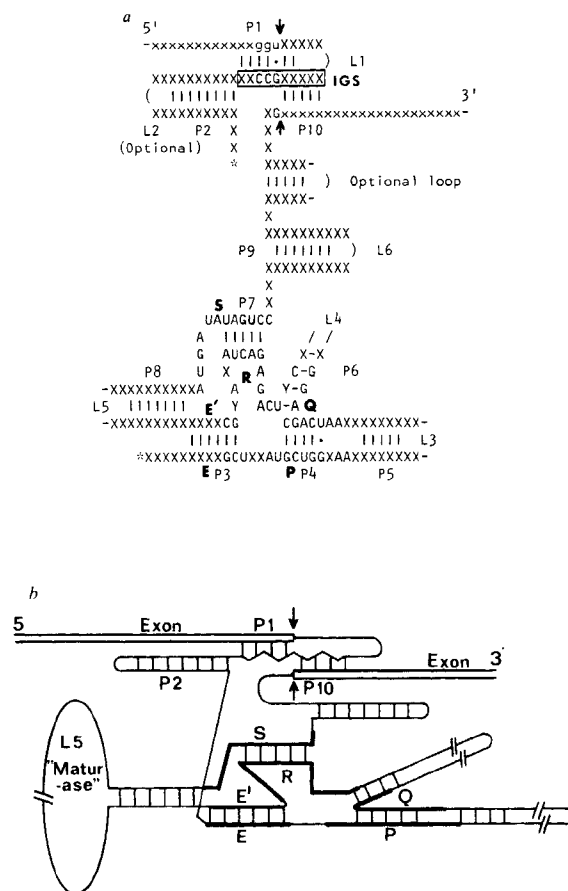
The extreme regularity and conservation of detail of RNA secondary structure within introns can be seen in Fig. 3a; all the conserved bases shown in the core structure in this figure are present in at least six out of eight introns and the conserved base pairs in all or seven out of eight introns. One of the most remarkable features that these introns have in common is the existence of a pair of sequences named by us E and E' (Table 1, Fig. 3). Two bases after the conserved sequence R, there is a conserved G base in all the introns followed by a C base in five out of seven introns. These two bases together with the next four (three in YC4, five in YOX4 and Y ω) make up the sequence we call E'. The amazing property of E' is that, although the last four bases are completely different from intron to intron, they and the GC preceding them are always capable of pairing perfectly with a sequence E that precedes sequence P by three bases (Figs 3, 6). This conservation of precise base-pairing ability of sequences in identical positions in all introns, independent of actual nucleotide sequence conservation, demands the interpretation that these sequences really do pair.

Further evidence for the functional importance of the RNA-RNA interactions between P and Q, R and S is provided by

looking at the effects on the pairings of variations from the consensus sequence. Sequences P and Q are strongly conserved between introns and species, but there are several variations from the consensus sequences. In all cases where a different nucleotide sequence is found within the section of P that can pair with Q, a compensatory alteration in sequence Q is found so that the base-pairing between the sequences is maintained. There are five such compensatory exchanges, the most striking being the case of NOX1, where an insertion of a base in P is exactly compensated for by two changes in Q. There are no alterations in the proposed base-pairing region of P that are not compensated for by complementary alterations in Q. The pairing regions of the R and S sequences are very precisely conserved; only in NOX3 does a change occur in part of the R sequence that pairs with S, and here too a compensatory alteration in S is found. All these compensatory exchanges in the variant sequences are shown in Fig. 4.

This model also accounts for the phenotypes due to several *cis*-acting mutations affecting splicing which have been sequenced by Slonimski and co-workers^{9,12}. The mutations fall into two clusters, which in YC4 have been called *box9* and *box2*. All the *box9* mutations that have been sequenced (including the equivalent class in YOX4) fall in R or E', and reduce the strength of base-pairing either of R and S or of E and E'; as R and E' are separated by only one base, they have previously been considered to be part of one functional sequence, whereas we propose that they have different roles, although both are involved in secondary structure formation. Details of the sequenced mutations are given in refs 9 and 12, and their effects on the R-S and E-E' interactions are shown in Fig. 5. Two mutations of the *box2* class which affect sequence S of YC4 have also been sequenced⁹. Both W300-3 and G2590 alter G (ref. 8) to A; this would destroy a central G-C base pair in the pairing of S with R (Fig. 5). Thus, our proposal also explains the observed phenotype of *box2* mutations. Weakening of the R-S or E-E' pairings by these mutations will reduce the stability of the core structure, thus preventing efficient

Fig. 3 Model RNA secondary structure for a class of fungal mitochondrial introns. *a*, A generalized secondary structure model derived from the RNA sequence of eight introns (YC3 data were not used), showing conserved bases and conserved pairings between non-conserved bases. *b*, A diagrammatic representation of the secondary structure showing only the key components. For convenience of two-dimensional representation, the RNA structure has been shown in *a* as if cut between P2 and P3 (or just 5' to E)—the arbitrary ends that are left are marked with an asterisk and the sequence is continuous between these points. Intron bases are in capitals, exon bases in lower-case letters. A, U, C or G is written when these bases occur in a particular position in at least six out of eight introns, while Y indicates a pyrimidine and R a purine base in six out of eight introns; otherwise X or x signifies any base. Except for P1 and P10 (see Fig. 6) when a base pair is given, it occurs in at least seven out of eight introns in this position. When the end of a stem-loop structure is closed by a round bracket, the number of bases shown is that of the consensus intron; when the end is shown with a dash after each nucleotide, then a large variable number of bases is found in the loop (see Table 1). Base-paired regions are labelled P1 to P10, loops L1 to L6. L5 contains the maturase-coding region of the majority class intron; the putative maturase-coding region of YOX3 occurs in L1, and this loop is also large (though non-coding) in NOX1. YOX3 and NOX1 have small L5 loops, all others have small L1 loops of the average size shown in the consensus structure. P3 is formed by the pairing of P and Q, P7 by the pairing of R and S, P1 and P10 by the pairing of IGS with exon sequences. The central core of the structure is P3, P4 and P7 with the associated P5, P6 and P8. P2 and P9 are peripheral stems which bring the splice sites close to the folded central core; P1 and P10 form the precise alignment structure. In *b* exons are shown as open bars, conserved sequences as thick solid lines, IGS as a wavy line, and base pairs are shown where conserved. The line joining P2 to E is of necessity out of scale, and represents only four bases on average. The L5 loop is shown as coding for the maturase protein as in YC4, but it can code for non-maturase protein (Y ω), be defective for protein coding (YOX4, NOX3), or very small (NOX1, NOX3).



formation of the precise alignment structure. This is why mutations within an intron cause splicing defects.

No mutations have so far been isolated in P, Q or E. This is probably because the genetic map is not saturated, that is, only a small proportion of all the possible mutations affecting splicing have been identified. Most of the mutations that have been isolated in R, S and E' are leaky¹³, so if point mutations in P and Q had slightly weaker effects they could be difficult to detect. Alternatively, the mutations that have been isolated may affect not only RNA-RNA pairing but also alter the specific binding sites of proteins involved in splicing, and the P-Q pairing might not bind any protein at all. The model predicts that the distribution of small deletion mutations would be much more widespread and include P and Q, that point mutations will be found in E, and that E mutations will be found as suppressors of E' mutations and vice versa.

The precise-alignment structure and the internal guide sequence

At some stage of splicing the splice sites must be aligned precisely and held together long enough for the excision of the intron and the ligation of at least the exon ends to be achieved. Although this could conceivably be done by proteins alone, a very reliable and precise way of aligning two nucleotide sequences is by way of complementary base-pairing with an adaptor molecule^{14,15}. We propose that this class of fungal mitochondrial intron RNAs possesses an internal guide sequence that functions as an adaptor, pairing with upstream and downstream exon sequences adjacent to the splice sites in such a way as to place the splice sites together exactly. The base-pairing in this precise-alignment structure need not be very extensive because we propose that the previous folding of the RNA core structure strongly favours the pairing of the internal guide sequence with the exon RNA sequences. In fact, most of the precise-alignment structures shown in Fig. 6 involve base-pairings (P1, 3–6 bp; P10, 3–8 bp) that are comparable with those proposed for the pairing of the U1 external guide RNA with intron sequences in the alignment of the ends of nuclear mRNA introns^{14,15} without previous folding.

Intron	Variant in pairing region
	PQ Pairings
Consensus	5' P 3' AUGCUGGAAA I I I I I GGACGCUAA 3' Q 5'
YOX3	AUGCgGGAAA I I I I I GGACGcUAA
NOX1	AUGCgUGGAA I I I I I GGACGuaUAA
NOX2	AUaCUGGAAA I I I I I GGAuGACUAA
Yw	uUGCUGaGAu I I I I I cGACGaaUAA
	RS Pairings
Consensus	5' S 3' AAGAUUAGUCC I I I I I ACAUCAGAGACU 3' R 5'
NOX3	AAGAUaaAGUCC I I I I I AuuUCAGAGACU

Fig. 4 The P-Q and R-S pairings with variants. Consensus pairings and all known exceptions are shown, demonstrating the conservation of base-pairing in the variant introns. Bases that differ from consensus are shown in lower-case, consensus bases in capitals. Intron terminology is given in Fig. 1 legend.

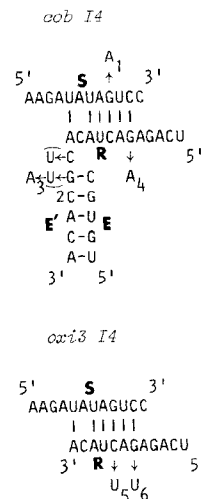


Fig. 5 Mutational evidence for the R-S and E-E' pairings. The R-S pairing of YOX4 and the R-S and E-E' pairings of YC4 are shown with all the sequenced *cis*-acting mutations affecting splicing^{9,12}. Numbers next to bases in mutants indicate the identity of the mutant^{9,12,13}: 1 = G2590 and W300-3; 2 = M4474 (a double base change); 3 = M7832; 4 = V353; 5 = G2190; 6 = G192.

The internal guide sequences are given in Table 1, and the IGS-exon pairings making up the precise-alignment structures are shown in Fig. 6; these correspond to P1 and P10 of Fig. 3. A similar though slightly shifted version of an alignment structure was proposed for Yw by Bos *et al.*¹⁶. If P1 is considered in isolation it involves an extra 1 or 2 bp beyond the U · G pair shown as the end of P1 in Fig. 6; these base-pairings are between the first bases of the intron and one or two of the IGS bases that can also take part in the P10 pairing.

The first argument in favour of this structure is the regularity with which it can be formed by a sequence (IGS) between the 5' splice site and sequence E. There are several conserved features, which can be located in Fig. 3 and seen in detail in Fig. 6. The finding that the precise-alignment structures are variable apart from these features agrees well with the considerable specificity of splicing reactions; note that the alignment structures and IGS of YOX4 and YC4, which share the same splicing specificity¹³, are very similar.

The phenotypes resulting from two known mutations can be explained if the mutations reduce the strength of the pairings in the precise-alignment structure. G2457 is a mit⁻ mutation of yeast that is clearly defective in the excision of YC4, but has been shown by DNA sequencing to be a single G → A transition affecting the last but one nucleotide of the upstream exon⁹; this G → A change would significantly reduce the stability of the pairing, as well as affecting the potential protein-binding site. This G and its pairing with a C in IGS within the P1 pairing are strongly conserved. The other example is the class of mutations known as ωⁿ, which occur in the gene for the large mitochondrial rRNA of *S. cerevisiae*; ωⁿ mutations remove the strong polarity (biased transmission of parental genotypes to offspring) that is otherwise found in crosses between ω⁺ and ω⁻ strains of *S. cerevisiae*. ω⁺ and ω⁻ strains differ in the presence and absence of the Yw intron, and ωⁿ mutations only occur in ω⁻ strains, never in ω⁺ strains. ωⁿ mutations have been shown by DNA sequencing⁸ to be a G → A transition three bases before the end of the upstream exon. This would reduce the strength of the P1 pairing in our model. Clearly, if this prevents RNA splicing, ωⁿ mutations could never occur in ω⁺ strains because the Yw intron would not be excised and this would be lethal.

The proposed structure thus fulfils all the criteria for a structure having a critical role in determining the precision of the splicing reaction. As for P, Q and E, we expect mutations to be found in the IGS and further mutations to be found in the

exon sequences near splice junctions as the genetic map becomes saturated.

Two introns which definitely have the core structure but only weak precise-alignment structures differ from the seven other introns in having extra DNA present between at least one splice junction and the core structure sequences (Fig. 1). The core structure of these introns is still necessary (hence its conservation) but not sufficient for bringing the ends of the intron together. There are two ways of interpreting the structure of NOX1 and YOX3. First, these introns are now spliced in a different way from the other seven, and either a completely new recognition mechanism has been superimposed or intron excision occurs in multiple steps, one of which may correspond to the standard model. Second, they are still spliced in the

standard way, the extra DNA contributing additional secondary structure to correct for its own presence.

Proteins and the splicing reaction

The model deals with the role of RNA sequences in the splicing process, and not with the role of proteins or the mechanism of the reaction itself. It demonstrates, however, that proteins are probably involved in stabilizing RNA secondary structure as well as in the cutting and ligation steps. Splicing proteins may also need to recognize parts of the secondary structure instead of, or as well as, sequences near the splice junctions.

The form of the precise-alignment structure has consequences for the mechanism of the splicing reaction. IGS bases have two alternative pairings as shown in Fig. 3a. Once the core structure, perhaps aided by proteins, allows the P10 pairings to form, the two forms of the structure must exist in an equilibrium due to branch migration unless one is favoured by the local environment. If the fully formed P10 pairing is present for a reasonable fraction of the time, or is stabilized by proteins, then simultaneous cuts could be made at the 5' and 3' cut sites in a structure which has both the cut sites at the junction of a very short (half-turn) helical segment with single-stranded RNA, and separated only by the length of a phosphodiester bond.

It is important to make the two cuts spatially and temporally close together so as to reduce the error rate of the reaction, and this can be achieved if recognition and cutting of the 5' and 3' ends only occur efficiently once the whole structure has formed. Given this proviso, a sequential mechanism is possible with a nick first occurring at the 5' scission site in the top strand of the full P1 pairing of Fig. 3a; the last base pair or two of P1 then dissociate or are displaced by P10 branch migration, and the 3' cut is made. Even a fully sequential mechanism is possible if P1 is stabilized by proteins after the 5' cut has been made. In certain nuclear mutant¹⁷ and *cis*-acting mutant¹⁸ strains of yeast, the 5' cut can occasionally be made independently of the 3' cut.

A strong body of evidence¹³ supports the involvement of intron-coded mRNA maturases in the excision of some of these introns. These could be important at any stage in the splicing process, including the endonuclease reaction. One specific problem of mitochondrial RNA splicing is that RNA synthesis and splicing and protein synthesis occur in the same compartment in mitochondria, so that the RNA being spliced is simultaneously being translated. The hypothesis that ribosomes have a positive role in splicing^{9,13} has received a little experimental support¹⁹. Ribosomes are likely to disrupt any secondary structure that does form, and in most of these introns ribosomes must cross the 5' splice junctions to synthesize the maturase. A positive role for ribosomes within the framework of our model might be to inhibit premature 5'-end cutting by disrupting the P1 pairing, although there are alternative ways of achieving this. However, ribosomes travelling into the intron will tend to disrupt the core structure. We have assumed that ribosomes are sufficiently well spaced to allow the splicing complex to form (ribosomes within loops should have little effect). If ribosomes are closely spaced, one of the roles of proteins binding to the core structure may be to prevent the disruption of the structure by ribosomes. In this case it is possible that the maturase is involved in stabilizing the structure against disruption by ribosomes, as well as acting as at least a specificity factor in the splicing reaction.

Conclusions

The class of fungal mitochondrial introns which we have considered possess, according to our proposal, all the specific elements required for their own excision. The model presented here accounts for all the data at present pertaining to the precise excision of these introns, and makes clear predictions of classes of mutations that should be found as well as suggesting the

Intron	EE' Pairings	IG/Exon Pairings
YC4	5' E 3' UGUGC 11111 ACACG 3' E' 5'	5' Exon -uacuuuaggu+ 1111 3'-UAUCCGGUUAUAAAC 111 -G ₊ cuccugauaac- Exon
YOX4	AUUUUC 111111 UAAAAAG	-auucuuaggu+ 111 -UAUCCGGUGACAAAC 111 -G ₊ caccucgaaguau-
YOX3	CAACAC 111111 GUUGUG	-gguuuaggu+ 111 -GCCUAGAAUUAUUAU- 111 111 111 -G ₊ aacuaauuuuacca-
YC3		-gguggguu+ 11111 -UACCUAGGAGAUUAUUAU 111 1111 -G ₊ cucagauucaaccc-
NC	UCCUGC 111111 AGGACG	-ggagguuu+ 11111 -CCAAGUAGACACAUA 11111 -G ₊ cucuguaauuauagc-
NOX1	CUUCGC 111111 GGAGCG	-cagauuaggu+ 11111 -UGCCGUAAUUAAGACCU- 111 -G ₊ guuuuccuagauuaa-
NOX2	UUUGGC 111111 AAACCG	-uuagccggu+ 11111 -CGGCCGUCAAAGAUACAAA 111 111111 -G ₊ gguaugcuaugguu-
NOX3	CGUUGC 111111 GCAACG	-accagaggu+ 11111 -UCUUCGGAUUAUAAAC 111111111 -G ₊ uuuuuuuuuuuuu-
Yw	AUUGGGUG 1111111 UAACCCGC	-gcuaggguu+ 11111 -CCUGUUCGCCCAUUAUAA 11 11111 -G ₊ aacaggguuuuuuag-

Fig. 6 Base pairing between the internal guide sequences and exon sequences adjacent to the 5' and 3' splice sites, and base pairing between E and E' in nine fungal mitochondrial introns. Intron bases are in capitals, exon bases in lower-case lettering. The precise alignment structures are shown for convenience as if both the 5' and 3' cuts have been made. The intron bases that were in the L1 loop (Fig. 3a) are shown as a linear array in the middle line with the first (5') base of the intron on the right, except where a dash indicates that a large number of bases are present 5' to the sequence shown. Arrows indicate the cut sites. The last (3') base of the intron is shown to the left of the 3' cut site because this G is a conserved feature of the precise alignment structure. The one or two IGS bases that can pair either in P1 or P10 (Fig. 3a) are thus shown in their P10 pairing mode.

possible types of activity of proteins involved in splicing. In this model there can also be no ambiguity in the splicing of multiple introns within one RNA precursor molecule, as the dependence of the formation of the splicing complex on previous folding of the intron RNA ensures that the 5' and 3' ends of the same intron are always chosen for the excision reaction.

This mechanism of RNA splicing may be of more general importance, because at least one nuclear intron, that in the gene for the large nuclear rRNA of *Tetrahymena*, fits this model precisely (R.B.W. *et al.*, in preparation).

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in the field, but particularly Professor Slonimski and his group whose precise genetic analysis of mitochondrial RNA splicing provided essential evidence. This work was supported by MRC Hproject grant G7904290CB to R.W.D. and C.S.

Note added in proof: One prediction of the model has already been fulfilled: Dr P. S. Perlman and co-workers (personal communication) have obtained a mutation in E that blocks splicing of YC4. Dr R. Schweyen and co-workers (personal communication) have obtained the same base change in E as a revertant of a *box9* mutation in E', and the double mutant has regained an intact pairing region. Computer-aided analysis of intron RNAs of *S. cerevisiae* and *Kluyveromyces thermotolerans*²⁰ has provided RNA structures in excellent agreement with the consensus core structure presented here.

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Characterization of a regulatory region upstream of the *ADR2* locus of *S. cerevisiae*

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We have used in vitro mutagenesis to identify a DNA sequence that is required for glucose regulation of the ADR2 locus of Saccharomyces cerevisiae. This region, located between 200 and 1,000 base pairs upstream of the structural gene for ADH II, has been identified by genetic and biochemical analysis as the site of ADR3, a cis-acting regulatory locus. If this region is deleted, ADR2 is no longer repressed by glucose. Moreover, if the sequence is excised and ligated in front of the ADC1 gene, it puts that gene under glucose control.

THE eukaryotic promoter has been identified by site-specific mutagenesis and deletion analysis of cloned genes¹⁻⁶. In several cases it has been possible to identify regions which have regulatory functions⁷⁻¹⁰, and occasionally, DNA sequences immediately 5' to the transcription initiation site have been implicated in regulating the expression of the adjacent structural gene. In other cases regulatory DNA sequences have been identified at a considerable distance from the affected gene¹¹⁻¹⁴.

We have chosen to examine the regulatory regions which affect the expression of the alcohol dehydrogenase (ADH) isozymes of the yeast, *Saccharomyces cerevisiae*. The 'classical' ADH I is synthesized during fermentative growth and is repressed about fivefold during growth on ethanol-containing medium¹⁵. A second isozyme, ADH II, is repressed when yeast are grown on glucose and is derepressed 100-fold when yeast are grown on a nonfermentable carbon source¹⁶. The structural genes coding for both ADH I (*ADC1*) and ADH II (*ADR2*) have been identified genetically¹⁸, cloned^{19,20} and their DNA sequences determined^{21,22}.

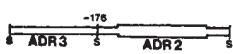
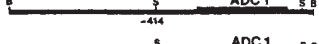
We show here that deletion of a specific 5'-flanking sequence of *ADR2* results in the loss of glucose control of ADH II synthesis. This sequence overlaps *ADR3*, a genetically defined cis-acting locus which regulates *ADR2*²³. In addition, we show that these sequences can be removed from their normal context in front of *ADR2*, placed upstream from the promoter of a different gene (*ADC1* in this case), and still retain their function.

Construction of an *ADR2* deletion

There is extensive nucleotide sequence homology in the region between the TATAA boxes and the transcription initiation sites of *ADC1* and *ADR2*²². The presence of an *SphI* restriction site immediately 5' to this homologous region in *ADR2* allowed us to test the hypothesis that regulatory sequences would be 5' to the *ADR2* TATAA box. If this idea were correct, a deletion of sequences 5' to the *SphI* site would prevent glucose repression of *ADR2*. To minimize gene dosage effects, the *ADR2* gene constructs were inserted into pBC3T1, a stable, single-copy vector containing a yeast centromere from chromosome 3 (*CEN3*)²⁴ and the *TRP1* gene¹⁷ (see Fig. 1). A 2.6-kilobase (kb) *BamHI/SphI* fragment containing *ADR2* and 1.2 kb of 5'-flanking sequence was cloned into pBC3T1 and designated pADR2. A second DNA fragment containing *ADR2* and only 176 base pairs (bp) of 5' sequence was inserted into the *SphI* site of pBC3T1. Clones with the fragment inserted in both orientations [pADR2(-176)R and pADR2(-176)L] were isolated.

These plasmids were used to transform the *trp*⁻ yeast strain 302-21 which contains defective alleles of all three ADH structural genes: *adc1-11*, *adr2-43* and *adm* (the last gene codes for a minor ADH isozyme found in the mitochondria). Single *TRP*⁺ colonies were isolated and grown on medium containing either glucose or ethanol. Cell extracts were prepared and assayed for ADH enzyme activity. As the results given in Table

Table 1 ADH enzyme activities of transformed yeast cells

	Plasmid	ADH activity (mU per mg)	
		Glucose	Ethanol
I		30	1600
		1200	2000
		1100	4100
II		10	300
		2200	1300
		4300	8300
III		10	200
		30	900
IV		2000	1300
		2300	1100

The *trp*⁻ yeast strain 302-21 was transformed with plasmid DNA according to the method of Beggs²⁹. *TRP*⁺ colonies were selected at random and assayed for enzyme activity in repression and derepression conditions using the following protocol. Cells from a stationary culture were inoculated into 10 ml *trp*⁻ minimal media containing 8% glucose at a concentration of $\sim 5 \times 10^5$ cells per ml. These cultures were grown overnight at 30 °C to a concentration of $\sim 2 \times 10^7$ cells per ml. One half of the culture was collected and assayed; the remaining half was washed once in H₂O and resuspended in 5 ml *trp*⁻ minimal media containing 3% ethanol. These cultures were grown for another 24 h at 30 °C and then collected. Assays were performed according to the method of Lutsdorf and Megnet¹⁶. Each value represents the average of the enzyme activities measured for three to five independent transformants.

1, section I, show, enzyme activity in strain 302-21 transformed with pADR2 is repressed on glucose-containing medium and derepressed on ethanol-containing medium. The amount of enzyme activity present in derepressed cells is comparable to that found for a strain containing a functional chromosomal *ADR2* gene (Table 2, strain 43-2B). Transformants of strain 302-21 containing an *ADR2* gene deleted to 176 bp 5' to the structural gene [pADR2(-176)L and pADR2(-176)R] have high levels of ADH (>1,000 mU per mg protein) even on glucose-containing medium. This is true for the fragment cloned in either orientation with respect to the vector, thus arguing against the likelihood that the expression observed is due to transcription initiated at a vector site with readthrough of the *ADR2* gene. This result strongly implies that the region that has been deleted in the plasmids pADR2(-176)R and pADR2(-176)L (that is, the region 5' to an *Sph*I site 176 bp upstream of the structural gene) contains sequences which are required for catabolite repression of *ADR2*.

Construction of an *ADR3/ADC1* hybrid

The identification of an *Sph*I site at -414 bp in the 5'-flanking region of *ADC1* allowed a further test of the hypothesis that the 5' *Bam*HI/*Sph*I fragment of *ADR2*, which we will designate *ADR3*, contains regulatory sequences. If this region is essential for catabolite repression, ligation of the *Bam*HI/*Sph*I fragment in front of *ADC1* should confer glucose repressibility on ADH I. The *Bam*HI/*Sph*I fragment was ligated to an *Sph*I/*Bam*HI fragment containing *ADC1*, and the hybrid was cloned into the *Bam*HI site of pBC3T1 and designated pADR3/*ADC1*(-414). To compare the expression of this hybrid gene with that of *ADC1* containing its own 5'-flanking sequences, two other plasmids were also constructed. A 3.5-kb fragment containing *ADC1* and 2,000 bp of 5'-flanking sequence was cloned into pBC3T1, and designated pADC1(-2000). An *Sph*I fragment containing *ADC1* and only 414 bp of 5'-flanking sequence was cloned in the same vector. This construction, pADC1(-414), was tested to ensure that a deletion of sequences 5' to -414 bp (the *Sph*I site) did not affect ADH I synthesis.

ADH activity was measured in cell extracts prepared from strain 302-21 transformed with the plasmids just described. Insertion of the *ADR3* sequence 5' to the *ADC1* gene [pADR3/*ADC1*(-414)] dramatically reduces ADH activity when cells containing the plasmid are grown on glucose-containing medium (Table 1, section II). The enzyme activity increases after growth in derepressing conditions to about 20% of the value obtained in extracts of cells transformed with pADR2. Strain 302-21 transformed with either pADC1(-2000) or pADC1(-414) has abundant enzyme activity when grown on either glucose- or ethanol-containing medium. The enzyme activities found for pADC1(-2000) are comparable to values found in strains with a functional chromosomal *ADC1* gene. These results show that the repression observed with the hybrid gene, pADR3/*ADC1*(-414), is due to the *Bam*HI/*Sph*I fragment normally flanking *ADR2*.

To identify which ADH isozyme is expressed in these transformed strains, cell lysates were analysed by electrophoresis on a non-denaturing polyacrylamide gel and stained for ADH activity (Fig. 2). Only ADH II is detected in cell extracts prepared from yeast transformed with pADR2 and the plasmids containing deletions 5' to *ADR2*. However, the isozyme present in cell extracts prepared from the strain transformed with the hybrid pADR3/*ADC1*(-414) is the *ADC1* gene product, ADH I. This proves that the repression and derepression of ADH activity that occur in cells transformed with the hybrid plasmid is not due to the inadvertent isolation of a transformed strain containing wild-type *ADR2*.

Construction of hybrids using *ADC1* 5' deletions

Derepression of *ADC1* in the hybrid construction pADR3/pADC1(-414) is weak compared with that observed for pADR2. The efficiency of derepression might be influenced by the distance of the *ADR3* sequence from the promoter.

Table 2 ADH expression in strains containing regulatory mutations that affect *ADR2*

Plasmid	ADH activity (mU per mg)							
	Strain: 526-3 (<i>ADR2 ccr1</i>)		302-21 (<i>adr2 ADR1</i>)		SF6-4A-M4 (<i>adr2 ADR1-5'</i>)		500-16 (<i>ADR2 adr1</i>)	
	Glc	EtOH	Glc	EtOH	Glc	EtOH	Glc	EtOH
pADR2	20	47	30	1,600	360	5,600	47	1,100
pADR3/ <i>ADC1</i> (-414)	NT	NT	10	300	43	1,400	30	2,500
pADR3/ <i>ADC1</i> (-150)	9	15	30	900	170	3,100	8	1,300
pBC3T1 (vector)	1	10	3	11	7	10	2	16
	Strain: 526-3 (<i>ADR2 ccr1</i>)		43-2B (<i>ADR2 ADR1</i>)		R234 (<i>ADR2 ADR1-5'</i>)		500-16 (<i>ADR2 adr1</i>)	
	Glc	EtOH	Glc	EtOH	Glc	EtOH	Glc	EtOH
Genomic <i>ADR2</i>	1	10	15	1,850	414	5,400	1	7

The enzyme activities of strains 43-2B and R234 are according to Ciriacy²⁵. All other activities were measured as described in Table 1 legend. NT, not tested.

Therefore, *ADC1* clones with deletions 3' to the *SphI* site were constructed and tested for their ability to express ADH I. Compared with a cloned *ADC1* gene with 2,000 bp of 5'-upstream sequence [pADC1(-2000)], deletion of 5'-flanking sequence to within 150 bp of the beginning of the structural gene has no effect on the level of *ADC1* expression when yeast containing these plasmids are grown on glucose-containing medium (results not shown).

The result that deletions of yeast DNA between 414 and 150 bp 5' to the *ADC1* gene did not reduce *ADC1* expression enabled us to test the hypothesis that *ADR3* function in a hybrid construction is sensitive to its position relative to the *ADC1* promoter. The *BamHI/SphI* fragment (*ADR3*) was ligated closer to *ADC1* using the 5'-deleted plasmids pADC1(-220) and pADC1(-150), which are deleted to within 220 and 150 bp 5' to *ADC1*, respectively. These hybrid gene constructions are designated pADR3/*ADC1*(-220) and pADR3/*ADC1*(-150).

These plasmids were used to transform strain 302-21; the transformed colonies were tested for enzyme activity and the results are shown in Table 1, section III. Ligation of the *ADR3* sequences 100 bp 5' to the TATAA homology region (pADR3/*ADC1*(-220)) resulted in no increase of derepressed enzyme activity relative to the original hybrid construction. However, strain 302-21 transformed with pADR3/*ADC1*(-150), in which this sequence is only 30 bp 5' to the TATAA homology region, has threefold higher ADH activity. The enzyme activity is ~60% of that found in the strain containing the cloned *ADR2* gene, pADR2.

These results demonstrate that the efficacy of the *ADR2* operator, *ADR3*, for the promotion of transcription of the adjacent gene during derepression depends on its placement with respect to the promoter of that gene. To test this idea further, a hybrid gene was constructed such that the *ADR3* region was located far upstream (~1,300 bp) from the TATAA sequence of *ADC1*. This plasmid [pADR3/*ADC1*(-1400)] and a control plasmid containing only *ADC1* sequence [pADC1(-1400)] were used to transform strain 302-21.

The enzyme activities of cell extracts of transformants are shown in Table 1, section IV. The activities found for yeast

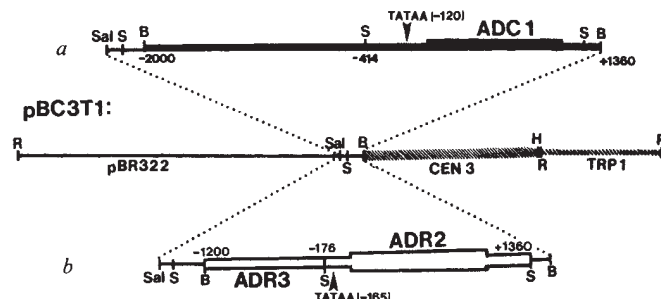
containing either pADC1(-1400) or pADR3/*ADC1*(-1400) are nearly identical to those found for yeast containing pADC1(-2000) when the cells are grown in either repression or derepression conditions. It seems that the *ADR3* sequence exerts no effect on *ADC1* expression when it is ligated at a site 1,300 bp upstream from the *ADC1* promoter. However, it is possible that sequences upstream of *ADC1* in this construction interfere with repression.

Glucose control of *ADR2* and *ADR3/ADC1* in mutant backgrounds

Strain 526-3 (*adc1-11*, *ADR1*, *ADR2*, *ccr1*) fails to express ADH II enzyme activity when grown on ethanol-containing medium due to a mutation at the regulatory locus, *CCR1* (ref. 25). This strain was transformed with the plasmids pADR2 and pADR3/*ADC1*(-150), and the ADH activity in the transformants measured (Table 2). Extracts of strain 526-3 containing these plasmids did not contain significant ADH activity when cells were grown on ethanol-containing medium. Therefore, *CCR1* function is required for derepression of enzyme synthesis for both pADR2 and pADR3/*ADC1*(-150).

ADR1 is another unlinked regulatory locus which affects *ADR2* expression. The semi-dominant *ADR1-5^c* mutation has two effects: it results in partially constitutive ADH II synthesis, and the ADH II activity is increased to a level higher than is found in *ADR1* wild-type cells during derepression²⁵. A strain containing the *ADR1-5^c* mutation, SF6-4A-M4 (*adc1-11*, *adr2-43*, *adm*), was transformed with pADR2 and hybrids pADR3/*ADC1*(-414) and pADR3/*ADC1*(-150). The enzyme activities of extracts from the transformed strains are given in Table 2. The enzyme activities shown for pADR2 correspond to those found in a strain containing a functional chromosomal copy of *ADR2* (Table 2, strain R234). The enzyme activities of cells transformed with the hybrid plasmids pADR3/*ADC1*(-414) and pADR3/*ADC1*(-150) are significantly elevated for both repressing and derepressing growth conditions relative to the levels found in a host strain containing wild-type *ADR1*. Again, the enzyme activities found in yeast containing pADR3/*ADC1*(-150) are higher than in

Fig. 1 Restriction map of vector pBC3T1 and yeast sequence inserts for pADC1(-2000) (a) and pADR2 (b). The vector pBC3T1 was constructed by first ligating a *HindIII/BamHI* fragment containing the yeast centromere *CEN3*²⁴ into the *HindIII* and *BamHI* sites of pBR322. Restriction enzymes and T4 DNA ligase (BRL) were used according to manufacturers' instructions. Methods for preparative isolation of DNA fragments have been described elsewhere²⁰. Plasmids from *E. coli* transformed to ampicillin resistance were analysed after extraction according to a method described by Holmes and Quigley²⁰. The yeast *TRP1* gene was isolated from the yeast-*Escherichia coli* shuttle vector YRp7 by *EcoRI* digestion and ligated into the *EcoRI* site of the *CEN3*-pBR322 vector after treatment of the *EcoRI*-cut plasmid with calf intestine alkaline phosphatase (Boehringer). The orientation of the *TRP1* gene in pBC3T1 is the opposite of its orientation with respect to pBR322 sequences in YRp7. Plasmid pADR2 was constructed using a *SalI/SphI* fragment from YRp7-*ADR2*-BSb which contains a 2.7-kb insert of yeast sequences containing the *ADR2* structural gene²⁰. This plasmid was digested with *SalI* and partially with *SphI*. A fragment containing 2.6 kb of yeast sequence including *ADR2* and 1,200 bp of 5'-flanking sequence was ligated into pBC3T1 cut with *SalI* and *SphI*. This construction has a direct repeat of ~200 bp of pBR322 sequences, which flank the yeast insert. Plasmid pADC1(-2000) was constructed by isolating a 3.5-kb *BamHI* fragment containing *ADC1* and 2,000 bp of 5'-flanking sequence from YRp9T6¹⁹. This was inserted into the *BamHI* site of pBC3T1. To construct pADR2(-176)L, in which yeast sequences 5' to the *ADR2* *SphI* site at -176 are deleted, plasmid pADR2(-1700) was partially digested with *SphI* and religated. Plasmids in which the upstream sequences were deleted were identified by restriction digestion. To prepare pADR2(-176)R, which is a plasmid containing a deleted *ADR2* fragment cloned in the opposite orientation as pADR2(-176)L, an *SphI* fragment containing the *ADR2* gene and 176 bp of 5'-flanking sequence was isolated. This fragment was cloned into pBC3T1 that had been cut with *SphI* and treated with calf intestine alkaline phosphatase. The orientation of the inserted fragment was determined by restriction enzyme analysis. The plasmid pADC1(-414), in which the sequences 5' to the *SphI* site at -414 are deleted, was constructed in a similar manner to that described for pADR2(-176)L. A series of hybrid genes were constructed in which the *ADR3* regulatory region was ligated to a variety of sites 5' to the *ADC1* gene and cloned in pBC3T1. Plasmid pADC(-414), with a deletion to the *SphI* site at -414 of *ADC1*, was reconstructed to form a hybrid gene (pADR3/*ADC1*(-414)) by replacing the *BamHI/SphI* fragment upstream of *ADC1* with the 1-kb *BamHI/SphI* *ADR3* regulatory region. The plasmid pADR3/*ADC1*(-150) was constructed using an *ADC1* gene isolated from pADC1(-150), which contains a *SalI* site ~150 bp 5' to the *ADC1* structural gene. An *ADR3* fragment was prepared from a construction in which the *SphI* site had been altered to a *XhoI* site using molecular recombination linkers (Collaborative Research). The 1-kb *BamHI/XhoI* *ADR3* region and the *SalI/BamHI* *ADC1* fragment were ligated into pBC3T1 cut with *BamHI* and treated with calf intestine alkaline phosphatase. Isolates in which the orientation of the structural gene with respect to the vector was the same as in pADC1(-2000) were identified by restriction analysis. The hybrid-gene plasmid pADR3/*ADC1*(-220) was constructed in the same manner, using *SalI/BamHI* fragment from pADC1(-220) in which a *SalI* site is 220 bp 5' to *ADC1*. The same method was used for the construction of pADR3/*ADC1*(-1400) using an *ADC1* fragment from a construction in which a *HindII* site, 1,400 bp 5' to the structural gene had been converted to a *XhoI* site using molecular recombination linkers. This fragment was also used to construct pADC1(-1400), by cloning it into pBC3T1 cut with *SalI* and *BamHI*. Restriction enzyme cleavage sites are *BamHI* (B), *SphI* (S), *SalI* (Sal), *EcoRI* (R) and *HindIII* (H).



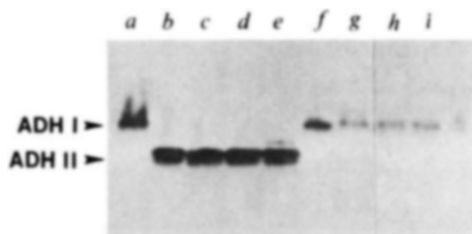


Fig. 2 Identification of the ADH isozyme expressed in transformed yeast cells. Cell extracts were electrophoresed on a non-denaturing polyacrylamide gel as previously described¹⁹, and stained for ADH activity²⁸. Lane *a* contains an extract from strain SF4-1B, which expresses only ADH I. Lane *b* contains an extract from strain R112, which expresses only ADH II. Lanes *c*–*i* contain extracts of strain 302-21 transformed with the following plasmids: *c*, pADR2; *d*, pADR2(–176)R; *e*, pADR2(–176)L; *f*, pADC1(–2000); *g*, pADR3/ADC1(–414); *h*, pADR3/ADC1(–220); *i*, pADR3/ADC1(–150).

yeast containing pADR3/ADC1(–414), in which the *ADR3* region is displaced further upstream relative to the *ADC1* promoter.

The recessive *adr1-1* mutation results in a very low level of *ADR2* derepression when cells are grown on ethanol-containing medium²³. The plasmids described above were used to transform strain 500-16, which is defective for *ADR1* (*adr1-1*, *adc1-11*, *ADR2*, *adm*). Table 2 gives the ADH activities of these transformed strains and reveals the surprising result that the *ADR2* gene on a plasmid is abundantly expressed in derepressing conditions despite being in a host strain in which the chromosomal copy of *ADR2* is not expressed due to the *ADR1* defect. The hybrid pADR3/ADC1 plasmids also express ADH activity in abundance. In this case, pADR3/ADC1(–414) is more fully derepressed than in a wild-type *ADR1* background.

Analysis of cell extracts by electrophoresis detected only ADH I activity in the pADR3/ADC1 transformants, proving that the chromosomal copy of *ADR2* is not derepressed concomitantly with the plasmid genes. Independence of *ADR1* is not due to the absence of upstream control sequences in pADR2, as three multi-copy plasmids containing the *ADR2* gene with 1, 6 and 13 kb of 5'-flanking sequence are all derepressed in a strain containing the defective *adr1-1* allele (results not shown).

Discussion

Two different lines of evidence suggest that the promoter for transcription of *ADR2* can be separated from its operator region, *ADR3*. First, deletion of yeast sequences upstream from an *SphI* site 176 bp 5' to the coding region of *ADR2* allows constitutive synthesis of ADH II on glucose. Second, when these upstream sequences are ligated to *ADC1*, this gene becomes glucose repressible. Because the presumed TATAA box for *ADR2* transcription is located 160 bp 5' to the coding region of *ADR2*, we conclude that all the sequences essential for efficient transcription of *ADR2* are located between the TATAA box and the initiation site of transcription, which is 54 bp 5' to the coding region of *ADR2*.

The operator region, defined as the DNA sequences which can confer glucose repressibility on either *ADR2* or *ADC1*, extends upstream from approximately position –176 for not more than 1 kb. This same region overlaps the *ADR3* locus which was identified by *cis*-acting regulatory mutations that allow partially constitutive ADH II synthesis on glucose medium²³. Most of these *ADR3^c* mutations are caused by Ty insertions between positions –210 and –125²⁶. Two non-Ty induced *ADR3^c* mutations have been located by cloning and DNA sequence analysis at position –220 (D. Russell, personal communication). Thus, there is a good correspondence between the genetic locus, *ADR3*, and the DNA fragment that confers glucose repressibility.

The function of the *ADR3* region depends on its position with respect to the promoter of the adjacent gene. Glucose repression mediated by *ADR3* is effective when the fragment is as far as 300 bp upstream from the TATAA box of *ADC1*. In contrast, efficient derepression of the hybrid gene occurs only when *ADR3* is ligated within 30 bp of the promoter. The evidence that hybrid genes can be constructed in which derepression of *ADC1* is incomplete but repression is apparently normal suggests that repression and derepression might occur by independent mechanisms.

The repression and derepression conferred by the *ADR3* locus contained on plasmids is subject to some of the same regulation as is the chromosomal *ADR3* region. Derepression of the hybrid gene pADR3/ADC1(–150) requires *CCR1* function. *CCR1* is a pleiotropic effector which is required for the derepression of several glucose-repressed enzymes, including *ADR2*²⁵. The hybrid gene also shows appropriate response (that is, increased enzyme activity) to *ADR1-5^c*, a regulatory mutation that results in partially constitutive synthesis of ADH II²⁵. However, plasmids containing *ADR3* do not require *ADR1* for expression of the neighbouring structural gene (either *ADC1* or *ADR2*) as does the chromosomal *ADR2* locus. This result is not understood.

The identification of glucose control sequences for *ADR2* and the effect of operator–promoter separation on gene activity may help explain how transposable elements alter *ADR2* expression. The ADH II-constitutive phenotype resulting from Ty insertion may be a consequence of the displacement of the operator far enough upstream of the promoter to negate its effect, as proposed previously^{26,27}. This hypothesis is consistent with the failure of *ADR3* to show any control over *ADC1* when it is ligated 1,300 bp upstream from its promoter, as in pADR3/ADC1(–1400). Secondary mutants of Ty-insertion *ADR2* mutants in which the Ty element has been replaced by a single 340-bp delta sequence have negligible ADH II activity when grown on glucose-containing medium and are only weakly derepressed²⁷. In these mutants the position of *ADR3* with respect to the TATAA sequence is similar to that found in the hybrid pADR3/ADC1(–414). Cells transformed with this hybrid also have enzyme activities that are low when grown in either repression or derepression conditions.

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LETTERS TO NATURE

A new class of radio pulsars

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Sufficiently low magnetic field neutron stars which accrete for long times from a surrounding keplerian disk can be spun up to millisecond periods. After accretion ceases, such stars could become isolated or binary pulsars, depending on whether their companions have subsequently disrupted the binary by either their tidal break ups or their supernova explosions. Such pulsars constitute a new class, with short periods, long apparent ages ($P/\dot{P} \geq 10^8$ yr) and pulsed optical, X-ray and γ -ray fluxes significantly below those expected for canonical pulsars with similar periods. Because of their long subsequent spin-down lifetimes, such pulsars would probably be observable if their birth rate exceeded even 10^{-4} that of the canonical ones. We propose here that the recently discovered millisecond pulsar 4C21.53 belongs to this new class, together with the binaries PSRs 1913+16, 0820+02 and 0655+64, as well as possibly several isolated pulsars such as PSRs 1952+29 and 1804-08. The possibility of such a class of fast accretion-spun-up pulsars has also been noted by Backus *et al.*¹

Several kinds of observations support the view that many neutron stars in the close accreting binaries which constitute the galactic bulge X-ray sources have weak ($B < 10^{11}$ G) surface magnetic fields: (1) analyses of post-burst cooling of X-ray bursters imply that accretion has been effective over the entire neutron star surface with no important effects from magnetic funnelling to polar regions²⁻⁵; (2) in such neutron stars there is no sign of the modulation of X-ray emission expected from rotation of a magnetized stellar surface with $B > 10^{10}$ G (ref. 6); (3) good agreement with theoretical models for burst properties is achieved when magnetic funnelling is not effective ($B < 3 \times 10^{11}$ G)⁷. In addition, various scenarios which would be expected occasionally to lead to such binaries imply some very low field neutron stars⁸. Thus, for an accreting 'non-magnetic' white dwarf⁹ ($B < 10^5$ G) which grows beyond the Chandrasekhar limit and collapses to a neutron star, simple flux conservation alone would be expected to amplify B to at most 10^9 G. Finally, theoretical models offer various reasons for expecting strong reductions in (dipolar) magnetic fields in many or most old ($> 10^7$ yr) neutron stars which cause them to be extinguished as pulsars^{10,11}.

There are a variety of scenarios for systems in which a neutron star accretes from a keplerian accretion disk initially fed by a companion^{8,12}. First, low mass X-ray binaries (galactic bulge sources). Typical accretion rates are 10^{-9} – $10^{-10} M_{\odot} \text{ yr}^{-1}$ for 10^9 – 10^{10} yr so that a mass $\geq 10^{-1} M_{\odot}$ is transferred to the neutron star. Because of orbit shrinking due to gravitational radiation¹² the ultimate fate of such binaries must be a single neutron star or black hole. The low mass primary, even if it became a white dwarf first, will ultimately overflow its diminishing Roche lobe and be partly accreted onto the neutron star with the remainder dispersed in space. In some cases the accret-

ing neutron star may become massive enough to collapse to a black hole, but often the remainder should be an isolated neutron star, embedded in the dilute remnant cloud of that part of the companion which was not accreted. For circular low mass binaries this last stage of evolution will take $< 10^{10}$ yr if the initial binary period is of the order of 5 h or less¹³. For highly elliptical orbits such as that of the binary pulsar PSR 1913+16 this can be accomplished with longer initial periods. (We note that even a double neutron star binary such as PSR 1913+16 must ultimately end as a single rapidly spinning neutron star or black hole.)

Second, high mass X-ray binaries where mass transfer is very rapid ($\sim 10^6$ yr) into the Roche lobe of the neutron star. Much of this mass may ultimately form a keplerian disk before accretion onto the neutron star. The primary may then evolve to a supernova which disrupts the binary or leaves behind a bound collapsed companion. Neutron star accretion from a relatively massive partially filled Roche lobe may well continue long after the primary has disappeared if the Eddington limit rather than disk viscosity is controlling the accretion.

When the accretion region nearest to the neutron star is a keplerian disk the star will spin up until the keplerian angular velocity at the disk inner edge (the stellar magnetosphere's Alfvén radius) approximately equals that of the star. When enough matter is accreted to achieve this steady state, the equilibrium period^{12,14,15} is

$$P \sim (6 \times 10^{-4} \text{ s}) B_8^{6/7} R_6^{18/7} (M/M_{\odot})^{-5/7} \dot{m}_{17}^{-3/7} \quad (1)$$

where $\dot{m}_{17}$ is the accretion rate in units of 10^{17} g s^{-1} , B_8 the surface dipole magnetic field in units of 10^8 G, M is the neutron star mass and R_6 is its radius in units of 10^6 cm. In estimating spin-up rates we shall neglect the torque on the magnetized neutron star from currents which flow through it as a result of the termination of its plasma-filled co-rotating magnetosphere at the Alfvén radius by conducting matter of different angular velocity. These may significantly change the spin-up and the dipole moment orientation but not equation (1). The accreted mass needed to achieve this limiting period is then

$$m_a \sim (0.22 M_{\odot}) I_{45} (10^3 P)^{-4/3} (M/M_{\odot})^{-2/3} \quad (2)$$

where I_{45} is the neutron star moment of inertia in units of 10^{45} g cm^2 , and the required accretion time is

$$T_a \sim 1.4 \times (10^8 \text{ yr}) I_{45} (10^3 P)^{-4/3} (M/M_{\odot})^{-2/3} \dot{m}_{17}^{-1} \quad (3)$$

The above equations are valid only if B is not so weak that the Alfvén radius shrinks to the stellar surface, that is, $B_8 \geq 0.9 (M/M_{\odot})^{1/4} R_6^{-5/4} \dot{m}_{17}^{1/2}$; otherwise

$$P \sim 2.7 \times 10^{-4} \left(\frac{M}{M_{\odot}} \right)^{-1/2} \left(\frac{m_a}{M_{\odot}} \right)^{-1} R_6^{1/2} I_{45} \quad (4)$$

Immediately after accretion ceases the spun-up neutron star will have a spin-down rate $\dot{P}$ and a lifetime T_s of a freely rotating pulsar:

$$\frac{P^2}{T_s} \equiv P\dot{P} \sim \frac{8\pi^2 R^6 B^2}{3Ic^3} \quad (5)$$

This gives an initial

$$\dot{P} \sim (3.2 \times 10^{-20}) I_{45}^{-1} (10^3 P)^{4/3} (M/M_{\odot})^{5/3} \dot{m}_{17} \quad (6)$$

and initial

$$T_s \sim (10^9 \text{ yr}) I_{45} (10^3 P)^{-1/3} (M/M_{\odot})^{-5/3} \dot{m}_{17}^{-1} \quad (7)$$

During its radio pulsar phase the spun-up neutron star thereafter has increasing P and $\dot{P}^{-1}$ related by equation (5).

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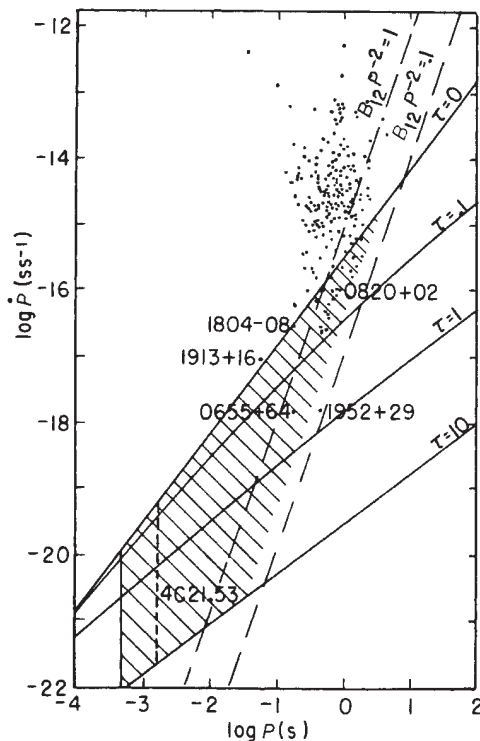


Fig. 1 The $\dot{P}$ - P diagram for pulsars, after refs 1 and 17. Dots mark observed values with units as marked. Constant age curves for the new class of accretion spun-up pulsars are shown with $\dot{P}$ values given in units of $I_{45}^{-1} (M/M_{\odot})^{5/3} \dot{m}_{17} s s^{-1}$. Their pulsar age τ is given in units of $(10^3 \text{ yr}) I_{45} (M/M_{\odot})^{-5/3} \dot{m}_{17}^{-1}$. After birth at some point on the birth curve ($\tau = 0$) a pulsar is assumed to move on a $\dot{P}\dot{P} = \text{constant}$ line; at age τ it is at the intersection of that line and the curve labelled τ . On hitting the appropriate $B_{12} P^{-2} = \eta$ line ($\dot{P}$ is in units of $R_6^2 I_{45}^{-1} s s^{-1}$ on these lines) it will die out as a pulsar. Thus, all pulsars of the new class should lie in a region similar to the shaded region of the diagram, bounded by the $\tau = 0$ line, the $B P^{-2}$ line, the $\tau \sim 10$ (age of the Universe) line and the line of rotational break-up of neutron stars. These pulsars are confined to that region even when the pre-birth spin-up did not reach the $\tau = 0$ curve (that is, accretion stopped at P longer than equation (1)). It seems that many of the known neutron star binary X-ray pulsars would not become radio pulsars if accretion were to be interrupted now; and some will never become radio pulsars even with continuing spin-up if they are now rotating close to their equilibrium period of equation (1). The possible theoretical ($\dot{P}$, P) points for 4C21.53 lie on the dotted line.

Typical observed accretion rates onto neutron stars in binaries are $\dot{m}_{17} \sim 1$. Almost all radio pulsars^{16,17} have $\dot{P}$ larger than initial values from equation (6) followed by spin down (unless $\dot{m}_{17}$ exceeds the Eddington limit by a typical three orders of magnitude). The new class of accretion spun-up pulsars would thus lie in a distinct region of the $\dot{P}$ - P plane, bound by the initial conditions of equation (6) and corresponding roughly to observed $\dot{P} < 10^{-17} s s^{-1}$ (Fig. 1). We consider it very significant that this region contains all known binary pulsars as

pointed out by Backus *et al.*¹ (Table 1), because binary radio pulsars with collapsed companions are the most promising candidates for old, low B neutron stars which have been spun up by accretion disks¹⁸. In Table 1 calculated initial $\dot{P}$ for $\dot{m}_{17} = 1$ are compared with observed $\dot{P}$ for these stars. Another potential candidate is the recently discovered radio pulsar 4C21.53 (ref. 19) for which a measured $\dot{P}$ is not yet available and for which there is no direct evidence supporting a present or past binary nature. We believe a history of past spin up by an accretion disk of a low B neutron star may be especially attractive because of the difficulty in understanding this star as a canonical young rapidly spinning pulsar. There appear to be problems in understanding an observation of a millisecond pulsar unless the neutron star had been relatively cold while it was being spun up and has also had a particularly low B ever since. Arguments for low B include the following: (1) A canonical B would give such a rapid spin down that the mere observation of such a pulsar would imply both an extraordinary birth rate and a very large pulsar population with periods between 10^{-3} and 10^{-1} s. We note also that no young supernova remnant is visible. (2) Such rapid spin down ($T_s \sim 1-10^2$ yr) would give internal heating from various mechanisms (crustal flow, superfluid vortex unpinning) that such a neutron star at Crab pulsar distances should have been readily observable by the Einstein Observatory. Observations, however, limit its surface temperature to $< 1.5 \times 10^6$ K (D. Helfand and R. H. Becker, personal communication). (3) It would be difficult to account for the absence of observed energetic high energy emission. A spinning neutron star may be expected to have a total current flow and a maximum achievable potential drop along open field lines proportional to $B P^{-2}$. For the spun-up neutron star

$$\frac{B_{12}}{P^2} = (1.8 \times 10^2) (10^3 P)^{-5/6} R_6^{-3} (M/M_{\odot})^{5/6} \dot{m}_{17}^{1/2} \quad (8)$$

For $P \sim 10^{-3}$ s and Eddington limit accretion $\dot{m}_{17} \sim 10$, $B_{12} P^{-2} \sim 600$, essentially the same as that of the Vela pulsar and less if the spin-up accretion rate was less than Eddington. There is then no compelling reason to believe that the high energy emission supported by such currents should exceed those from Vela. A millisecond spun-up pulsar which resembled Vela but was further away than the Crab pulsar would then certainly be presently undetectable in pulsed optical, X-ray and also in pulsed γ -ray emission below 30 MeV. In higher energy γ rays it may be observable because the period is determined. If $B_{12} \sim 1$, equation (8) would imply a value of $B_{12} P^{-2}$ almost 10^6 that of Vela. (4) It seems difficult for pulsar models which put the source of radiation within the magnetosphere to give GHz radiation when the velocity of light cylinder is near the stellar surface unless the surface B is small. Such models generally utilize e^+ pair production and/or relativistic ion flows. In either case a necessary condition is $B_{12} P^{-2} \geq n$, with n of order unity for e^+ pair production and $10^{-1}-10^{-2}$ for pure ion flow models. In either case we see from equation (8) that the inequality is easily satisfied for $P \sim 10^{-3}$ s. However, in most models, relevant plasma frequencies are sufficiently high throughout the magnetosphere that expected coherent radio emission is generally at frequencies much above 1 GHz.

If B is indeed to be anomalously low there is an additional reason that a cold, accretion, spin-up scenario is preferred to

Table 1 The binary pulsars and the superfast pulsar as accretion spun-up pulsars with $\dot{m}_{17} = 1$, $M = 1 M_{\odot}$ and $I_{45} = 1$

Pulsar	P (ms)	$\dot{P}_{\text{theory}} (s s^{-1})$	$\dot{P}_{\text{observed}} (s s^{-1})$	B (G)	T_s (yr)	T_a (yr)
PSR 1913+16	59	7.3×10^{-18}	8.6×10^{-18}	2.1×10^{10}	2.6×10^9	6.0×10^5
PSR 0820+02	865	2.6×10^{-16}	1×10^{-16}	4.8×10^{11}	1.0×10^8	1.7×10^4
PSR 0655+64	196	3.6×10^{-17}	$< 5 \times 10^{-18}$	8.6×10^{10}	1.7×10^8	1.2×10^5
4C21.53	1.5	5.5×10^{-20}	?	2.9×10^8	8.9×10^8	8×10^7

$\dot{P}_{\text{theory}}$ is calculated from equation (6) on assuming that the presently observed P is still the initial one. Because, in fact, both P and $\dot{P}^{-1}$ are expected to increase roughly by equation (5), one expects $\dot{P}_{\text{theory}} \geq \dot{P}_{\text{observed}}$.

a hot, supernova, one. Even if a high spin, low magnetic field pulsar could be formed in a supernova (and thus retain a high spin for times much longer than its cooling time), the large heat flux from that hot neutron star may still generate sufficiently large crustal magnetic fields by magnetothermal effects¹⁰. The neutron star would then spin down before the B field dissipated and thus have a period far longer than a millisecond. By contrast, in the presence of ohmic dissipation, the much smaller stellar heating during the accretion phase need not generate large magnetic fields¹⁰.

We note that the predicted initial $\dot{P}$ of 4C21.53 as a superfast spun-up pulsar is $<5.5 \times 10^{-20} \text{ s s}^{-1}$ ($I_{45}^{-1} (M/M_{\odot})^{5/3} \dot{m}_{17}$). A larger $\dot{P}$ could indicate the presence of non-electromagnetic, for example, gravitational radiation torques.

The expected lifetime as X-ray sources of the low mass galactic bulge close binaries has been estimated to be of order 10^9 yr. If a large fraction do indeed end up as the single spun-

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Persistent X-ray emission from a γ -ray burst source

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A quiescent X-ray source detected with the Einstein X-ray Observatory in a location consistent with that of an intense γ -ray burst¹⁻³ is shown here to be also consistent with the location of the 1928 optical transient⁴, the likely optical counterpart of the γ -ray burst source GBS0117-29. The probable maximum X-ray luminosity is only $\sim 10^{31} \text{ erg s}^{-1}$ and supports the hypothesis that either γ bursts are produced by accretion instabilities^{5,6} or, if due to published models for a thermonuclear flash⁷, they recur with intervals ≥ 50 yr. Such long recurrence times may be inconsistent with limits inferred from both the γ burst observations⁸ and optical data⁴.

The interplanetary burst network timing of γ bursts has yielded several relatively precise error boxes for source locations. One of these, the $\sim 5 \text{ arc min}^2$ location¹ of the γ burst detected on 19 November 1978 (GBS0117-29) led to the discovery of its probable optical counterpart from a brief optical transient found on a 1928 Harvard patrol plate⁴. The γ burst location was sufficiently precise that a search for an accompanying X-ray¹⁻³ and radio⁹ counterpart could be carried out. A $\sim 9,000$ s X-ray guest investigation observation^{2,3} has been conducted with the IPC detector on the Einstein X-ray Observatory¹⁰ on 1-2 July 1980. Preliminary analysis of these data yielded a 3.5σ source detection with a relatively large location uncertainty in which the optical transient was later found. These X-ray data have been recently reprocessed using an improved analysis system with the result that both the source detection and its positional coincidence with the transient optical counterpart are established. Further studies of this X-ray source, as

up neutron stars of scenario (1) (many, perhaps, still in the dilute cloud formed by that part of the primary which was not accreted), the relative number of radio pulsars to neutron star X-ray sources would be of order $T_s/10^9 \text{ yr} \sim 1$. As there are of the order of 25 such neutron star X-ray sources⁶ we might then expect a similar number of fast accretion-spun-up radio pulsars in the Galaxy. Almost all will be well beyond the $\sim 6 \times 10^3$ light yr limit for pulsar detectability but to observe one or two does not seem implausible. Certainly it is among the galactic bulge X-ray sources that millisecond neutron star periods might be common.

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well as upper limits from Einstein observations of several other γ burst positions, are reported elsewhere¹¹.

The new source location is plotted in Fig. 1, together with the position of the optical counterpart⁴ and the original γ burst error box¹. The X-ray source error box has a 90% confidence radius of 45 arc s and is centred 42 arc s north-west of the optical counterpart. The X-ray flux detected in the 'hard' 0.5-3.0 keV band is $1.0 \times 10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$. From the recent determination of $\log N$ - $\log S$ for high latitude X-ray sources¹², the probability that an X-ray source with flux $\sim 1 \times 10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$ should be found within 1 arc min of the optical counterpart is only 1.7×10^{-3} . The consistency of the position of the persistent X-ray source with that of the optical transient link its identity to the larger source location region for the γ -ray burst. The 19 November 1978 burst was among the most intense ever recorded, which may account for the apparent absence¹¹ of other persistent X-ray counterparts (omitting the anomalous case of the 5 March 1979 burst apparently from the supernova remnant N49).

As the steady X-ray source was not detected in the 'soft' 0.15-0.5 keV band and no significant low energy absorption is expected at the -83° galactic latitude position it seems that the source is not a coronal emission source (for example, stellar X-ray emission from an M dwarf), but is instead an accretion source with a characteristically hard ($kT \geq 2 \text{ keV}$) spectrum. Too few counts were detected to allow useful spectral fits, hardness ratios, or low energy cutoffs to be determined from the data. A temporal analysis was carried out, however, and shows that the X-ray emission is consistent with a constant flux throughout the entire 9,000 s observation.

The probable optical counterpart of the 1928 optical transient and γ buster was detected⁴ with quiescent magnitude ≥ 23 on the B and V 4-m plates of M. Liller. However, deeper searches carried out a month later with a 1.3-m telescope and CCD camera¹³ gave upper limits of magnitude ~ 25 . Subsequent more sensitive CCD observations¹⁴ have detected the quiescent and possibly variable emission from the source at magnitude ~ 25 . Thus the optical flux appears to be variable by a factor of at least 10 on time scales no more than ~ 1 month. Presumably the quiescent X-ray flux is also variable although follow up X-ray observations are needed. The ratio of X-ray to optical luminosity is therefore poorly constrained but is probably in the range $L_x/L_{\text{opt}} \approx 6-60$. This is similar to that found for low-mass X-ray binaries where the optical light arises from an accretion disk rather than the non-degenerate stellar com-

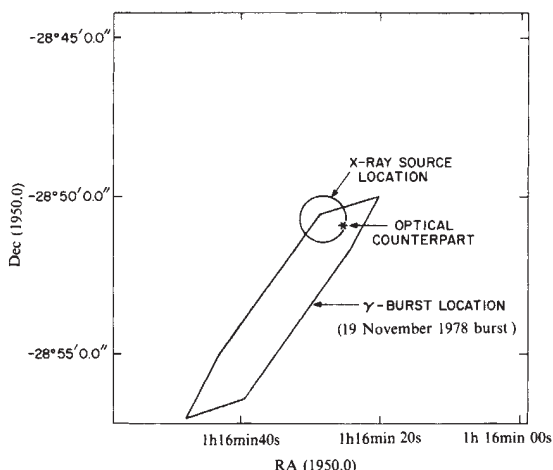


Fig. 1 Location (90% confidence error circle with 45 arc s radius centred at RA(1950) = 01 h 16 min 28.58 s, dec(1950) = $-28^{\circ}50'42''$) of the quiescent X-ray source which is the probable counterpart for the γ -ray burster GBS0117–29. The interplanetary network position for the 19 November 1978 γ burst¹ and the location of the 1928 optical transient⁴, the probable optical counterpart, are also shown.

panion. The L_x/L_{opt} value is probably not consistent with the value expected for an isolated neutron star accreting from an interstellar cloud but is intermediate between the typical values for compact binaries containing accreting degenerate dwarfs (with $L_x/L_{\text{opt}} \sim 1$) or accreting neutron stars (with $L_x/L_{\text{opt}} \sim 10^3$). Since the super-Eddington luminosities inferred for some γ bursts as well as the possible detection of both redshifted positron annihilation line radiation as well as possible cyclotron features all point strongly to a magnetized neutron star origin for γ bursts^{15–17}, we shall explore the consequences of this interpretation.

Our estimate for L_x/L_{opt} suggests that the system is underluminous (relative to compact binaries containing neutron stars) in X rays by perhaps a factor of 10. This could be due to the non-simultaneity of the X-ray and optical observations and/or to a highly variable accretion rate. Alternatively, it may indicate a relatively low accretion rate and a variable 'additional' optical contribution from cyclotron radiation in a $\sim 10^8$ G field at an effective Alfvén surface, where matter may accumulate before a burst. If the bursts are due to Rayleigh–Taylor like instabilities of $\sim 10^{19}$ g of matter (required for an $\sim 10^{39}$ erg total energy burst) accumulated in a magnetospheric shell, its radius would be $R \sim 2 \times 10^7$ for an assumed surface field $B \sim 1 \times 10^{12}$ G. The (assumed dipole) magnetic field in the shell would then in fact be $\sim 10^8$ G. Note that black-body radiation from the shell would not dominate the optical cyclotron radiation if the thermal temperature of the magnetospheric shell is $T \leq 4 \times 10^4$ K. Note also that such a 'shell' must, in fact, be a disk-geometry (a thick ring in the accretion disk?) since its large column density ($\sim 10^{28}$ cm $^{-2}$) would be opaque to quiescent X rays from the central neutron star.

The observed X-ray flux would require (for an assumed maximum distance of 1 kpc and $L_x = 10^{31}$ erg s $^{-1}$) an accretion rate of $\approx 1 \times 10^{-14} [d(1 \text{ kpc})]^2 M_{\odot} \text{ yr}^{-1}$. This value is probably too low to be consistent with published nuclear flash models for γ bursts^{7,16} unless the distance is substantially greater than ~ 1 kpc or the burst recurrence time is ≥ 50 yr, the accretion rate is highly variable, or further theoretical work on flash models shows that flashes yielding $\sim 10^{39}$ erg can be produced with a total accumulated mass of only $\sim 10^{20}$ g (as required for a ~ 5 – 10 yr recurrence time at the derived accretion rate). Such a large distance would make GBS0117–29 a halo object, whereas the isotropy and size distribution for γ bursts in general suggests¹⁸ that they are an extended disk population in the Galaxy. Such a long recurrence time appears to be inconsistent with the detection of the optical burst⁴. A highly variable (factor of ≥ 50) accretion rate, while somewhat *ad hoc*, cannot be ruled

out, and it might, in fact, be suggested by the variable optical flux. Once again, repeated X-ray observations are needed. However, if the average accretion rate and X-ray luminosity vary only by factors of ~ 3 , as do most neutron star X-ray binaries (we may exclude X-ray transients, as their quiescent L_x/L_{opt} values are probably much lower than observed for this quiescent γ burster), we conclude that the accretion instability models^{5,6} for γ bursts may be preferred.

If the quiescent X-ray emission from GBS0117–29 is typical, the summed X-ray flux from all such objects could be significant. For a currently extrapolated total of ~ 150 bursts yr $^{-1}$ (ref. 19), and an assumed typical source distance of 300 pc and ~ 1 yr burst recurrence time, the volume density of γ bursters is $\sim 1.5 \times 10^{-6}$ pc $^{-3}$. This is within an order of magnitude of the value derived by Hertz and Grindlay²⁰ for low-luminosity ($\sim 10^{31}$ erg s $^{-1}$) accretion X-ray sources in the Galaxy as measured by the galactic plane survey carried out with the Einstein Observatory. Most of these low luminosity ($\sim 10^{31}$ erg s $^{-1}$) X-ray sources are probably cataclysmic variables but it is possible that some are the quiescent counterparts of γ burster sources. Given the similarities in space density, it is also possible that the γ bursters are a large fraction of the neutron star products of cataclysmic variable evolution wherein continued accretion onto a degenerate dwarf eventually violates the Chandrasekhar limit and collapse to a neutron star ensues. Such systems might also be expected to have evolved (by mass transfer) such low mass non-degenerate stellar companions that their absolute magnitudes would be consistent with the stringent limits ($M \geq 15$, assuming $d \leq 1$ kpc) recently derived^{13,14} for the GBS0117–29 source.

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The tide in the seas of Titan

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The surface temperature of Titan¹ is about 95 K, between the melting point (90.6 K) and the boiling point (118 K) of methane at the total surface pressure of 1.6 bar. Voyager 1 IR observations^{1,2} suggest a methane abundance in the lower atmosphere >0.19 of saturation. Comparison with the mean H₂O abundance in the Earth's atmosphere suggests¹ a CH₄ saturation level ≈ 0.5 . Thus the volume mixing ratio of methane is probably about 6% and methane begins to condense out some 40 km above the surface. It is therefore a natural speculation that

methane exists in the liquid state on the surface of Titan. The atmospheric CH_4 corresponds to a liquid layer ~ 10 m deep. But it is unlikely that the buffer for atmospheric methane would have a capacity equal only to that of the atmosphere itself. On Earth, the quantity of water in the oceans is on average several times 10^4 the quantity of water vapour in the atmosphere. Thus, if Titan has oceans of liquid CH_4 , they are probably >100 m deep. We argue here that, if this ocean exists, the present high eccentricity of Titan requires its depth to be >400 m, and that such an ocean can be detected by radar techniques.

The relief observed on other saturnian moons, such as Tethys and on jovian moons of comparable size, such as Ganymede, is ~ 1 km in scale³; we would anticipate comparable relief on the surface of Titan. Initially, therefore, we expect both land and sea on Titan. The interaction of UV light and charged particles with the titanian upper atmosphere produces a class of dark organic solids called tholins⁴⁻⁷, which are expected over geological time to coat the land and accumulate as submarine deposits to a depth also in excess of 100 m (ref. 8).

Saturn will raise a significant tide in the seas of Titan, but because the satellite is probably in synchronous rotation with a period of 15.95 days the tide will be near-stationary and centred close to the Saturnward radius vector. If the solid body of Titan has not relaxed to hydrostatic equilibrium, then the maximum height of the solid body tide will be^{9,10}

$$s = \frac{(5/2)(M_S/M_T)(R^4/a^3)}{1 + \tilde{\mu}[1 - (3\sigma/5\rho)]/[1 - (\sigma/\rho)]} \quad (1)$$

and the difference between the maximum heights of the oceanic and body tides will be

$$h = \frac{(M_S/M_T)(R^4/a^3)}{[1 - (3\sigma/5\rho)] + [1 - (\sigma/\rho)]/\tilde{\mu}} \quad (2)$$

where M_S and M_T are the masses of Saturn and Titan, respectively; R is the surface radius of Titan; a is the semi-major axis of Titan's orbit; $\sigma = 0.45 \text{ g cm}^{-3}$ is the density of the putative titanian ocean; $\rho = 1.88 \pm 0.01 \text{ g cm}^{-3}$ is the mean density of Titan³; and $\tilde{\mu} = 19\mu/2\rho gR$, where μ is the rigidity of the solid body of Titan and g is the surface acceleration due to gravity. The greater the rigidity, the greater will be the amplitude h of the apparent oceanic tide.

The mean density of Titan is intermediate between the uncompressed densities of ice and rock; the solid body of the satellite is likely to consist of an icy mantle and a rocky core. The rigidity of water ice at temperatures near 100 K is $4 \times 10^{10} \text{ dyn cm}^{-2}$ (ref. 11). For methane and ammonia ices and their clathrates, μ should not be significantly less. The rigidity of rock in the interior of Titan will be larger. The central pressure is bounded by the following inequality¹², which is independent of the equation of state: $P_0 \leq P_c \leq (\rho_c/\rho)^{4/3} P_0$ where $P_0 = 3GM^2/8\pi R^4$. Allowing the central density, ρ_c , to range as high as 5 g cm^{-3} , we find $33 \leq P_c \leq 118 \text{ kbar}$, a pressure range corresponding to that of the upper mantle of the Earth down to $\sim 350 \text{ km}$ depth, a region¹³ in which μ is $\sim 0.7 \times 10^{12} \text{ dyn cm}^{-2}$ and varies slowly with depth. Thus, we obtain a lower bound on μ and h by adopting the rigidity of water ice. Then, $\tilde{\mu} \geq 6$, and

$$h \geq 100 \text{ m} \quad (3)$$

for the amplitude of the apparent, near-stationary, oceanic tide on Titan. Tides generated by the other saturnian moons have sub-millimetre amplitudes.

The possible existence of an ocean on Titan requires us to reconsider the effects of tidal dissipation. Previous studies¹⁴ treat the effects of dissipation in the solid body of the satellite; but, as on Earth¹³, solid body dissipation may be negligible compared with oceanic dissipation. There is no direct evidence that Titan is in synchronous rotation, but the time scale to achieve synchronicity is estimated¹⁵ at 10^7 – 10^8 yr. The orbital eccentricity of Titan, $e = 0.0289$, is comparatively high. Tidal distortion decreases as the cube of the distance; thus, the

amplitude of the tide raised by Saturn on the satellite will vary by a factor of $3e$, or 9% over its 15.95-day period. Titan rotates such that one face is directed towards the empty focus of its orbit, whereas the tide raised on it by Saturn is directed towards the planet. Consequently, the tide oscillates across the face of the satellite. The energy loss associated with these oscillations could, over the age of the Solar System, result in significant damping of the orbital eccentricity. As there appear to be no mechanisms available to increase e , the observed eccentricity can be used to establish limits on the surface topography and oceanic depths of Titan.

If the solid body of Titan has relaxed to near-hydrostatic equilibrium, then the maximum height of the mean solid body tide is given by equation (1) with $\tilde{\mu} = 0$. We have $s \leq 250 \text{ m}$ where the deviation from the upper limit is determined by the degree to which Titan is centrally condensed: the tide height is 250 m only if the density of the satellite is uniform¹⁶. It would also follow for hydrostatic equilibrium that $h = 0$, and if the orbit of Titan were circular then the depth of a shallow ocean would be everywhere the same and independent of the phase of the orbital period. However, as we have already noted, the orbit of Titan is far from circular. Since all deformations are small and can be added linearly, and since for short period oscillations the rigidity of the solid body cannot be ignored, then, regardless of the degree of relaxation of the solid body, the amplitude of the apparent oceanic tide will vary by an amount $3eh$ ($\geq 9 \text{ m}$) over a 15.95-day period, where h is given by equation (3).

Because the effect on e of the tide raised on Saturn by Titan is negligible¹⁴, the time constant τ for the decay of the orbital eccentricity is given by^{14,17}

$$\tau = \frac{2}{21} \frac{M_T}{M_S} \left(\frac{a}{R}\right)^5 \frac{Q}{nk_2} \quad (4)$$

where n , k_2 and Q are the mean motion, the Love number and the specific dissipation function of Titan respectively. Thus, $\tau = 4 \times 10^6 Q/k_2 \text{ yr}$ and unless $Q/k_2 \geq 1,200$, τ is less than the age of the Solar System and the present comparatively high value of e is difficult to understand.

The Love number is determined by the internal density and rigidity distributions and can be written¹⁶ as $k_2 = (5/2)[H - (2/5)]$, where $H \leq 1$ and is unity only for a homogeneous satellite in hydrostatic equilibrium. Since $\tilde{\mu} \geq 6$ the distortion of Titan's solid body is small and H can be estimated¹⁶ from a point-core model as $H = (2/5)[1 - (3\sigma/5\rho)]^{-1}$. Substituting, we find $k_2 = 0.17$. Hence, for $\tau > 4,600 \text{ Myr}$, we must have

$$Q > 200 \quad (5)$$

If the putative titanian ocean is global in extent, then the above lower bound on Q effectively rules out the existence of large land masses, which, as on Earth, would act as barriers to the tidal flow and would generate low values of Q (~ 10). If the ocean cover is nearly complete, then Q may be large (>200), but only if the mean depth D of the ocean is also large so that oceanic boundary layer turbulence is weak. Following Goldreich and Soter¹⁸ we estimate that the rate of energy loss due to boundary layer turbulence is $\dot{E} = f\sigma\langle v^3 \rangle \text{ erg cm}^{-2}$, where $f \approx 0.002$ is the coefficient of skin friction and v is the local velocity of the tidal current. The time-dependent part of the tide-raising potential of Saturn at some point (R, θ, ϕ) on Titan is

$$V = -\frac{GM}{a} \left(\frac{R}{a}\right)^2 3e \times [\frac{1}{2}(3 \sin^2 \theta \cos^2 \phi - 1) \cos nt + \sin^2 \theta \sin 2\phi \sin nt] \quad (6)$$

The first term in equation (6) excites the radial tide due to the variation in Saturn's distance and the second term is due to the optical libration of Titan. We estimate that the average tidal flow due to the radial tide alone is given by

$$\langle v^3 \rangle^{1/3} = (2/105 \pi^2)^{1/3} (3eh/D)nR \quad (7)$$

where v^3 has been averaged over the surface of the satellite and over one tidal cycle. This estimate neglects the effects of coriolis forces, which act to increase the tidal flow rates; thus, our value of $\langle \dot{E} \rangle$ is an underestimation.

We set $\langle \dot{E} \rangle = ce^3$. Then, since the total orbital angular momentum of the system is conserved (the spin angular momentum of Titan is negligible), one can show that

$$\dot{e} = -ce^2/E \quad (8)$$

where E is the total orbital energy. As the initial eccentricity of Titan's orbit must be <1 , integration of equation (8) yields

$$|c| < |E|/et \quad (9)$$

where t is the age of the system. If we now write $\langle \dot{E} \rangle = x\langle \dot{E}_r \rangle$, where $\langle \dot{E}_r \rangle$ is the energy loss due to the radial tide alone, then on substitution we find that equation (9) is satisfied only if

$$D > 300x^{1/3} \text{ m} \quad (10)$$

We estimate that, for the combined radial and libration tides, $x > 2$ and that the effects of coriolis forces will act to increase this limit further. Hence, we conclude, conservatively, that

$$D > 400 \text{ m} \quad (11)$$

Equations (1) and (2) for the tide heights assume that the kinetic energy of the tidal flow is negligible compared with the potential energy: the tides are assumed to be in static equilibrium. This is the case for the oceanic tide only if $D \gg (1/10)(nR)^2/g = 10 \text{ m}$, a condition¹⁹ which our previous arguments suggest is satisfied on Titan. If $D \approx 10 \text{ m}$, then the kinetic and potential energies would be comparable and we would expect enhanced dissipation and a reduced Q due to resonance in tidal basins. This is a further argument for a deep ocean. The velocity of the tidal current will be $v = (h/4D) \text{ m s}^{-1}$, providing a possible source of erosion especially in shallows and on the equivalent of continental margins. It is therefore conceivable that the eccentricity-driven departure from a pure stationary tide has, over geological time, eroded away most of the land masses on Titan, and is thereby responsible for the large value of Q [equation (5)].

Accordingly, there seem to be two internally consistent possibilities: (1) a near-global methane ocean $>400 \text{ m}$ deep that has inundated or eroded away most of the land on Titan, and (2) almost no methane ocean at all. In the latter case, the position of the surface of Titan near the liquidus in the CH_4 phase diagram will have to be considered a distracting coincidence, and the observed atmospheric methane must maintain itself against photolysis by some mechanism other than the equilibrium vapour pressure resupply from a methane ocean⁸. Fortunately, the choice between these alternatives can be made by remote observations.

The dielectric constant, ϵ , at microwave frequencies of liquid methane at the surface temperature of Titan is²⁰ about 1.7. As the square of the imaginary part of the complex refractive index, $k^2 \ll (\sqrt{\epsilon} - 1)^2$, the normal-incidence Fresnel equation yields a microwave reflectivity for a smooth methane ocean of about 0.017. For many organic solids $\epsilon \approx 2.6$ at visible wavelengths, increasing to 4–6 at microwave frequencies. X- and S-band values of ϵ for tholins have not yet been measured, but are likely to be in this range. The corresponding normal-incidence Fresnel reflectivities, with the same condition on k , range from 0.05 to 0.18. Thus we expect a contrast between the microwave reflectivity of methane oceans and tholin-covered land ranging between a factor of several and a factor of 10. If the land is not entirely inundated and the solid body has not relaxed to near-hydrostatic equilibrium, then we might expect a systematically higher radar reflectivity near the 0° and 180° meridians. If the solid body has relaxed then we might expect small variations in the disk-integrated reflectivity at a constant central meridian longitude with a period of 15.95 days.

Preliminary attempts to observe Titan near elongation with the 305-m Arecibo telescope during the 1979 opposition set only an upper limit of unity to the radar reflectivity (D .

Campbell and S. Ostro, personal communication). Considerably higher precision can be expected during the next Arecibo apparition of Titan in the mid-1990s, which can also check whether Titan is in fact in synchronous rotation. These considerations make still more interesting recent proposals to examine Titan, from orbit or from a probe-carrying flyby bus, with a spaceborne radar reflectometer.

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Design of a 13% efficient n-GaAs_{1-x}P_x semiconductor-liquid junction solar cell

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We report here the design of the most efficient non-aqueous semiconductor-liquid junction solar cell studied to date. Our approach involves the use of ternary semiconductor electrodes made from solid solutions of a large band gap material, GaP, and a small band gap material, GaAs. We demonstrate here that photoanodes consisting of such materials are capable of simultaneously yielding high open circuit voltages and favourable wavelength response to the solar spectrum. A few n-type semiconductor-liquid junction solar cells in aqueous solutions have been reported to yield high ($>10\%$) solar-to-electrical conversion efficiencies^{1–3}. However, for most materials, rapid photoanodic corrosion dominates the interfacial photochemistry^{4–8}. Non-aqueous solvent systems can suppress electrode decay due to corrosion^{4,7,8}; but modest ($<6\%$) conversion efficiencies have been observed for all photoanodes studied in solar irradiation conditions^{9–13}. The photoanodes used here yield over 13% solar-to-electrical conversion efficiencies, or more than double the efficiency of any other non-aqueous semiconductor-liquid junction solar cell previously reported.

We have studied a set of compositions of GaAs_{1-x}P_x, $0 < x \leq 1$, obtained as epilayers deposited on n⁺-GaAs or n⁺-GaP substrates by vapour phase epitaxy techniques. The epilayer is thick enough (0.1 mm) to prevent any photoactivity in the substrate. This has been verified by the lack of photocurrent on excitation of GaAs_{1-x}P_x ($x \geq 0.3$) samples on n⁺-GaAs substrate with light $\lambda \geq 750 \text{ nm}$.

Composition of the epitaxial layers used was verified by electron microprobe analysis and found to be: GaAs_{0.85}P_{0.15}, GaAs_{0.72}P_{0.28}, GaAs_{0.61}P_{0.39}, GaAs_{0.38}P_{0.62}, GaAs_{0.31}P_{0.69}, and

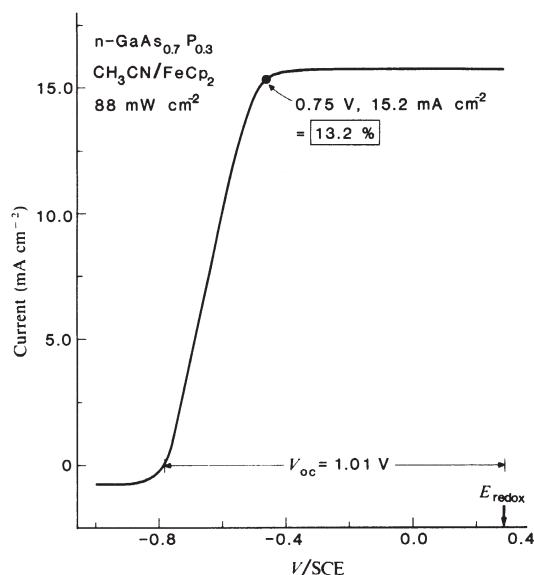


Fig. 1 Current-voltage curve at 50 mV s^{-1} (88 mW cm^{-2}) of a sample of $\text{n-GaAs}_{0.72}\text{P}_{0.28}$ in $0.1 \text{ M Fc}/0.5 \text{ mM Fc}^+/\text{ACN}$. The electrolyte is $1.0 \text{ M (Et}_4\text{N) BF}_4$, and the solution is stirred magnetically. The solution potential is $+0.22 \text{ V/SCE}$, and photocurrent negative of this potential represents net conversion of light into electricity.

$\text{GaAs}_{0.05}\text{P}_{0.95}$. Increasing the P content of the samples increases the band gap, E_g , in a monotonic, but not linear fashion, from 1.44 eV ($x = 0$) to 2.25 eV ($x = 1$) (ref. 14). A direct-to-indirect gap transition occurs at composition $x = 0.44$, with n-GaP having an indirect gap of 2.25 eV and a direct gap of 2.75 eV . Samples (0.1 cm^2) were mounted as photoelectrodes by conventional techniques. Ohmic contacts were formed by evaporation of In at 1×10^{-6} torr and annealing under N_2 at 400°C for 15 minutes. Potentials were recorded versus either a standard calomel reference electrode or a Pt wire placed $\sim 1 \text{ cm}$ from the working electrode. We have made no effort to minimize or instrumentally correct for the uncompensated ohmic resistance ($\sim 50 \Omega$) in this cell design; thus the efficiencies reported here are conservative by 10–20%. Light sources were either the Sun, a calibrated W-halogen ELH-type lamp with a ground glass diffuser, or a 5 mW He/Ne laser. Light intensities were measured with a Solarex secondary standard silicon cell and verified to within 10% by an Eppley thermopile. For samples with $E_g < 2.0 \text{ eV}$, we consistently find differences in efficiency measurements between our calibrated ELH lamp and actual sunlight of $< 10\%$. The samples were etched with 1:1 $\text{HF}/\text{H}_2\text{O}_2$ for 15 s, rinsed with H_2O , and air dried before use.

W-halogen irradiation (88 mW cm^{-2}) of a sample of $\text{n-GaAs}_{0.72}\text{P}_{0.28}$ (not deliberately doped; $N_D = 3.3 \times 10^{15}$) in the solution $0.1 \text{ M ferrocene (Fc)}$, 0.5 mM Fc^+ in dry acetonitrile (ACN) solvent (electrolyte $1.0 \text{ M (C}_2\text{H}_5)_4\text{N}^+\text{BF}_4^-$) typically yields the current-voltage profile represented in Fig. 1. We observe an open-circuit voltage, (V_{oc}), of 1.01 V , a short-circuit current of 15.7 mA cm^{-2} , and a fill factor of 0.73, leading to an optical-to-electrical conversion efficiency of 13.2% (15.2 mA cm^{-2} and 0.76 V at the point of maximum power). In natural sunlight (64 mW cm^{-2}), we observe similar behaviour, and conversion efficiencies of 12.5–13.0% are typical.

For comparison with other literature data, we have also recorded the current-voltage characteristics at 632.8 nm with a He-Ne laser. Values are consistent with the W lamp data, and at intensities of 20 mW cm^{-2} we observe over 22% efficiency for conversion of monochromatic 632.8 nm light to electricity.

The extremes of our series, n-GaAs and n-GaP, have been previously studied in ACN electrolytes. n-GaAs, with $E_g = 1.44 \text{ eV}$, is ideally suited for terrestrial solar applications; however, in similar non-aqueous cells, it is reported to yield modest operating voltages (0.25 V) (ref. 15), low quantum efficiencies (0.3) (ref. 16), and fill factors which decline at

irradiation levels above 0.01 suns^{15} , leading to solar conversion efficiencies of 2.4% (ref. 15). n-GaP is reported to yield output voltages of up to $0.8\text{--}1.0 \text{ V}$ (ref. 17). However, its E_g (indirect) of 2.25 eV leads to low short-circuit currents and poor solar efficiency. The goal of our studies with $\text{GaAs}_{1-x}\text{P}_x$ was to maintain the high output voltage characteristics of n-GaP with the favourable solar wavelength response of GaAs.

We observe much larger values of V_{oc} with $\text{n-GaAs}_{0.72}\text{P}_{0.28}$ than reported for n-GaAs. A comparison of our $\text{GaAs}_{1-x}\text{P}_x$ data with the performance of GaAs (refs 15, 16) suggests that substitution of P for As in the GaAs lattice eliminates most of the sources of inefficiency in ACN solvent. Consistently, we observe high values of the fill factor, > 0.70 for all samples with $x \geq 0.25$, which are fairly independent of light intensity even at irradiance levels near 1 Sun.

A plot of V_{oc} at 88 mW cm^{-2} in $\text{Fc}/\text{Fc}^+/\text{ACN}$ solution as a function of x (Fig. 2) shows that even for low levels of P, ($x = 0.3$), V_{oc} has increased to 1.01 V . Further increases in x lead to smaller changes in V_{oc} . As expected, the short-circuit currents measured with our W-halogen irradiation source decrease with increasing value of E_g (ref. 18). Thus, there is a maximum P content for optimum cell efficiencies, and for the range of samples studied, this occurs with $\text{GaAs}_{0.72}\text{P}_{0.28}$, $E_g = 1.77 \text{ eV}$. An E_g of 1.4 eV is optimum for AM1 solar conversion devices¹⁹, and increasing the E_g to 1.77 eV decreases the maximum theoretical AM1 conversion efficiency from 36 to 32% (ref. 19). However, for these $\text{GaAs}_{0.72}\text{P}_{0.28}$ samples, the increased values of both V_{oc} and the voltage at maximum power (V_{pmax}) compared with those reported for n-GaAs in non-aqueous systems results in much higher efficiencies for the larger E_g materials used here ($\text{n-GaAs}_{0.72}\text{P}_{0.28}$: $V_{oc} = 1.01$, $V_{pmax} = 0.76$; n-GaAs: $V_{oc} = 0.62$, $V_{pmax} = 0.25$).

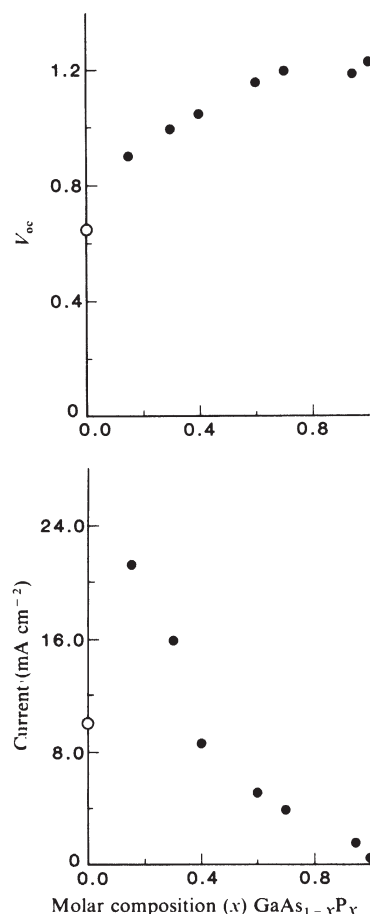


Fig. 2 Plot of open-circuit voltage and short-circuit current against composition for $\text{n-GaAs}_{1-x}\text{P}_x$. All measurements were performed in conditions identical to those in Fig. 1. $\circ$, Previously reported data for n-GaAs, taken from refs 15 and 16.

We have studied changes in V_{oc} for our n-GaAs_{0.72}P_{0.28} as a function of solution potential, E° , with a variety of redox reagents. As measured by either cyclic voltammetry or steady-state current-voltage curves, we find that V_{oc} is relatively constant (0.95 ± 0.10 V) for a range of redox couples whose E° spans more than 1.5 V. For example, we find that the photoanodic peak in a cyclic voltammogram (a measure of V_{oc})²⁰ for n-GaAs_{0.72}P_{0.28} appears at -0.71 V/SCE for Fc oxidation (E_p at Pt = $+0.30$ V/SCE) and for the leuco form of methyl viologen, MV[•], at -1.86 V/SCE (E_p at Pt = -0.85 V/SCE). Such behaviour has been observed for several semiconductor-liquid interfaces, and has been attributed to either carrier inversion at the surface²¹ or to a high density of surface states²² which act to pin the surface Fermi level to values independent of the solution E° . Such situations, as in non-aqueous n-GaAs systems,¹⁵ limit the maximum V_{oc} attainable and can yield lower than optimum conversion efficiencies. Substitution of P for As in the lattice of n-GaAs leads to stronger bonding in both the bulk and surface regions of the material. Initial increases in x thus produce a highly favourable shift in factors controlling V_{oc} , with only a slight increase in E_g . Further increases in x yield little improvement in V_{oc} , only serve to increase the value of E_g , and thereby lower the overall efficiency.

Deliberately dried ACN solutions (3-Å sieves) yield extremely reproducible ($\pm 5\%$) current-voltage parameters over periods of hours with no visible surface damage or deterioration of cell properties. As reported for n-GaAs in ACN, injection of small amounts of H₂O (for example 75 mM H₂O) leads to rapid photocurrent decay, presumably due to oxide formation¹⁵. At present, we have observed stable photocurrents ($\pm 10\%$) at 15 mA cm⁻² with n-GaAs_{0.72}P_{0.28} for passage of over 10³ C cm⁻² at short circuit with no apparent weight loss of the crystal or visible surface damage. We have ruled out substantial photocorrosion with experiments using thin (5 μm) epilayers of n-GaAs_{0.72}P_{0.28} on n⁺-GaAs. Assuming a six electron decomposition process, consumption of the entire epilayer in this 2.5 × 2.5 mm crystal would require ~1 C of charge. After passage of over 30 C through this particular crystal, we still observe the V_{oc} and wavelength response of the n-GaAs_{0.72}P_{0.28} epilayer with no apparent contribution from the substrate. Photocorrosion processes would thus appear to be minimal. Long-term experiments are in progress, and the ultimate stability of these systems will probably depend on the extent to which water can be excluded from the electrolyte, solvent, and cell environment.

We note that our 13% efficiencies have been obtained on electrode surfaces with no deliberate attempts to optimize fully donor densities, reflectivity losses, and surface treatments. Such procedures have been shown to improve the performance of several aqueous photoelectrochemical systems in conditions similar to those utilized here²³. We are developing such optimization procedures for our photoelectrodes, as well as studying the effect of ion implantation of P into the surface region of n-GaAs. The high efficiencies of the GaAs_{1-x}P_x system demonstrate that properly designed non-aqueous electrolyte based photoelectrochemical cells can provide efficiencies competitive with aqueous systems. Moreover, control of V_{oc} by varying bulk composition can be an important approach to the design of efficient semiconductor-liquid junction cells, as evidenced by the dramatic improvements in efficiency observed with our photoelectrode materials. At present, the 13% solar conversion efficiency for n-GaAs_{0.72}P_{0.28} system represents the highest value reported for any semiconductor-liquid junction cell in actual solar conditions.

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³⁶Cl bomb pulse measured in a shallow ice core from Dye 3, Greenland

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Nuclear weapons tests at oceanic sites during the 1950s produced a large amount of ³⁶Cl ($t_{1/2} = 3.0 \times 10^5$ yr) through neutron capture of ³⁵Cl in seawater. Part of this anthropogenic ³⁶Cl was injected into the stratosphere from where it was redistributed throughout the Earth. This pulse of ³⁶Cl was first detected in rainfall by Schaeffer *et al.*¹. The global deposition rates are several orders of magnitude larger than the natural pre- and post-bomb ³⁶Cl production rate that reflects cosmic-ray spallation of atmospheric ⁴⁰Ar. Because ³⁶Cl is not a fission product, the anthropogenic ³⁶Cl is produced mainly in marine tests carried out on small islands and barges where a large amount of seawater chlorine is present to serve as a target. This target element requirement leads to a temporal dependence different from the other bomb produced isotopes²⁻⁴. We have determined the temporal dependence of ³⁶Cl fallout by measuring the depth profile of ³⁶Cl in an ice core from Dye 3 Greenland (65° 11' N, 43° 50' W) using tandem accelerator mass spectrometry⁵. We show here that the results agree well with a calculation of the ³⁶Cl produced and injected into the stratosphere by the tests.

The samples were prepared from a 100-m ice core drilled at Dye 3 in 1980 as a side project of the US-Danish-Swiss Greenland Ice Sheet Program (GISP). Based on space charge measurements⁶, the core was cut at the site into pieces containing the precipitation of approximately 1 yr. The $\delta^{18}O$ profile determined later (W. Dansgaard, personal communication) showed that the actual intervals ranged from 0.5 to 1.5 yr (Table 1). The sample weights ranged from 0.9 to 2.2 kg. Before adding Cl carrier (1.52 mg Cl) and Be as carrier (for ¹⁰Be measurements that will be reported elsewhere) to the melted ice, an aliquot was taken for chloride analysis. The natural chloride content, measured by ion chromatography for several of the samples, was found to be about 50 μg per kg-ice and thus contributed only ~3% to the chloride content of the prepared sample. The chloride was separated by precipitation of AgCl and purified as described elsewhere⁷.

The ³⁶Cl concentrations were measured using the University of Rochester's MP tandem van de Graaf accelerator facility. A major improvement to the system since our previous ³⁶Cl

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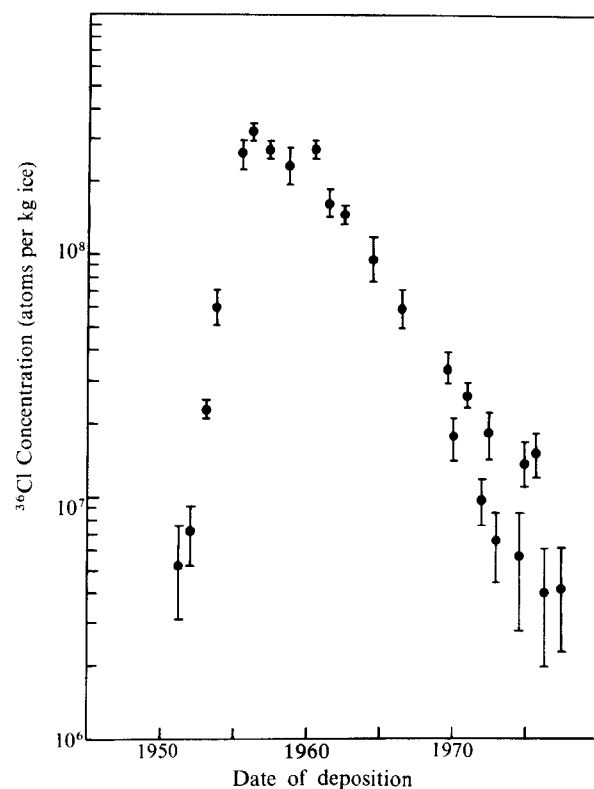
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Table 1 ^{36}Cl content of Dye 3 ice samples

Sample	Weight (kg)	Interval (yr)	Accumulation* (m)	Season-year†	$^{36}\text{Cl}/\text{Cl}$ ($\times 10^{-15}$)	Atoms per kg-ice ($\times 10^6$)
B1	1.50	1.25	1.23	A76-W77/78	250 $\pm$ 100	4.3 $\pm$ 2
B2	1.43	0.75	0.90	W75/76-A76	210 $\pm$ 100	4.0 $\pm$ 2
B3	1.17	0.50	1.16	S75-W75/76	680 $\pm$ 100	15.0 $\pm$ 3
B4	1.13	0.86	1.29	S/A74-S75	590 $\pm$ 100	14.0 $\pm$ 3
B5	1.29	0.88	1.30	A73-S/A74	280 $\pm$ 100	6.0 $\pm$ 3
B6	1.22	0.36	0.90	Sp/S73-A73	310 $\pm$ 100	6.7 $\pm$ 2
B7	1.25	0.88	1.50	S72-Sp/S73	880 $\pm$ 100	19.0 $\pm$ 3
B8	1.47	1.00	0.96	S71-S72	530 $\pm$ 100	9.6 $\pm$ 2
B9	1.45	1.00	0.59	S70-S71	1,480 $\pm$ 150	27.0 $\pm$ 3
B10	0.90	0.50	1.37	W69/70-S70	610 $\pm$ 100	18.0 $\pm$ 3
B12	1.09	0.75	0.89	Sp68-W68/69	1,430 $\pm$ 300	34.0 $\pm$ 7
B14	0.98	0.63	0.88	Sp/S66-W66/67	2,330 $\pm$ 350	62.0 $\pm$ 9
B16	2.20	1.13	1.18	W63/64-W/Sp65	8,130 $\pm$ 1,800	96.0 $\pm$ 22
B18	1.27	1.25	0.54	W61/62-S63	7,020 $\pm$ 700	150.0 $\pm$ 15
B19	1.73	1.00	1.05	W60/61-W61/62	10,800 $\pm$ 1,000	165.0 $\pm$ 17
B20	2.01	1.25	0.94	A59-W60/61	20,900 $\pm$ 1,500	274.0 $\pm$ 20
B22	1.65	1.00	0.90	A57-F58	14,700 $\pm$ 3,000	230.0 $\pm$ 50
B23	1.30	0.75	0.96	W56/57-A57	13,400 $\pm$ 600	272.0 $\pm$ 0.15
B24	1.33	1.00	0.61	A/W55-W56/57	16,700 $\pm$ 900	330.0 $\pm$ 20
B25	1.59	0.86	1.01	W54/55-A/W55	16,100 $\pm$ 1,600	267.0 $\pm$ 30
B26	1.90	1.00	0.90	W53/54-W54/55	4,400 $\pm$ 650	62.0 $\pm$ 9
B27	1.43	1.25	0.50	A52-W53/54	1,300 $\pm$ 100	24.0 $\pm$ 2
B28	1.63	1.25	0.88	S51-A52	470 $\pm$ 100	7.5 $\pm$ 2
B29	1.21	0.81	0.59	S/A50-S51	250 $\pm$ 100	5.4 $\pm$ 2

* Actual accumulation depth of ice.

† A, Autumn; W, winter; Sp, spring; S, summer.

**Fig. 1** The ^{36}Cl profile in an ice core drilled at Dye 3, Greenland, showing the large pulse that resulted from activation of ^{35}Cl during marine nuclear weapons tests. The age scale was determined by counting the seasonal variations in $\delta^{18}\text{O}$.

was kept small (final weights ranged from 0.5 to 1 mg Cl) to ensure that the $^{36}\text{Cl}/\text{Cl}$ ratios before and after the bomb pulse would be well above background levels. Even with the new ion source these small samples caused some difficulties with beam stability. This is reflected in the quoted 1σ uncertainties (Table 1) which range from 5 to 50% and are generally larger than statistical errors. The background level for this run was fairly high and variable because of contamination during the sample preparation or loading and/or cross contamination during the measurements. The addition of more carrier to reduce the $^{36}\text{Cl}/\text{Cl}$ for the samples at the deposition peak would have alleviated these problems. From the experience gained in this run and others⁷ we feel that 3–5% errors are possible with 2–3 mg Cl samples and that 1-kg ice samples should be adequate for measuring pre-bomb ^{36}Cl in polar ice cores.

The results given in Table 1 and Fig. 1 are expressed as atoms of ^{36}Cl per kg of ice and have not been corrected for the variable precipitation rate (Table 1). The $^{36}\text{Cl}/\text{Cl}$ ratios were reduced by the background value, which ranged from 70 to 200×10^{-15} , and were normalized to an NBS standard. The results show that ^{36}Cl concentrations at Dye 3 increased by a factor of more than 100 from pre-bomb levels. From 5×10^6 atoms per kg-ice in 1950, concentrations increased rapidly to 300×10^6 atoms per kg-ice in 1955 and then held that level until about 1960. Between 1960 and 1970 the ^{36}Cl decreased exponentially with a mean life of about 3 yr. Because of the high background and the lack of data points before 1950 we can only give an upper limit of 5×10^6 atoms kg^{-1} for the pre-bomb production rate for cosmogenic ^{36}Cl .

The integral of the ^{36}Cl bomb pulse (Fig. 1), when multiplied by an average accumulation rate for Dye 3 (ref. 10) of $50 \text{ g cm}^{-2} \text{ yr}^{-1}$, gives a total fallout at Dye 3 of 1.3×10^8 atoms cm^{-2} . This will now be compared with an estimate of the ^{36}Cl produced in oceanic tests and transported to Greenland. Table 2 lists the marine tests with a yield over 1 Mton that have been published^{11–13}, although we have not included the seven bombs >1 Mton detonated by the UK at Christmas Island (2°N , 127°W) during the Grapple series of tests because these were small air bombs whose contribution of ^{36}Cl to the strato-

measurements in polar ice^{7,8} is a new Cs sputter ion source⁹ designed specifically for tandem accelerator mass spectrometry. This source produces a high negative ion current (10–20 μA) with improved stability. For this project the quantity of carrier

Table 2 ^{36}Cl production in marine nuclear weapons tests

Test	Date	Explosive yield (Mton)	Location*	Predicted total ^{36}Cl production (kg)
Mike	31 October 1952	10.4	E	19.9
King	15 November 1952	>0.2	E	0.4
Bravo	28 February 1954	15	B	28.7
Zuni	27 May 1956	3.53	B	6.8
Tewa	20 July 1956	5.0	B	9.6
Koa	12 May 1958	1.37	E	2.6
Oak	26 June 1958	8.9	E	17.0
Castor	15 January 1968	0.5	T	
Canopus	24 August 1968	2.5	T	
Procyon	8 September 1968	1.0	T	
Dragon	30 May 1970	0.1–1	T	
Licorne	3 July 1970	~1	T	
Rhea	14 August 1971	~1	T	

* E, Pacific Proving Ground, Eniwetok Island (11° N, 162° E); B, Pacific Proving Ground, Bikini Island (11° N, 165° E); T, Tuamotu Archipelago (21° S, 137° W).

sphere was probably quite low. Also, the large USSR tests at Novaya Zemlya Island (75° N) during 1960–61 were not included. It is not known if these test sites were near the ocean, or whether they were air or surface tests. They do not appear to have contributed in a significant way to the total ^{36}Cl . The contribution of ^{36}Cl from surface tests would not be significant since the Cl content of soil and rock is two orders of magnitude less than that for seawater.

Assuming that 2×10^{26} neutrons are produced per megaton¹⁴, that half of the bomb neutrons enter the water, and that 32% of the neutrons that enter the water produce ^{36}Cl (ref. 15), we obtain the total ^{36}Cl produced from each premortatorium test (Table 2, column 5). For the simplest calculation, we can assume that all of this ^{36}Cl (85 kg) enters the stratosphere and is deposited uniformly over the Earth. This results in a global average deposition of 2.8×10^8 atoms cm^{-2} . This calculated accumulation is about a factor of two higher than our measured value at Dye 3. It is, however, well documented that the distribution of fallout is not globally uniform. Measurements of ^{90}Sr deposition between 1958 and 1966 show that the deposition rate between 60° N and 70° N is actually 1.3 times lower than the global average¹⁶. Correction for this nonuniformity reduces the discrepancy between measured and calculated ^{36}Cl deposition to less than a factor of two. We consider this to be excellent agreement since: (1) some of the ^{36}Cl produced may not enter the cloud; (2) ^{90}Sr may have a different particle size distribution from ^{36}Cl , (3) a site dependence of fallout within Greenland of a factor of two has been observed³, and (4) the effect of precipitation rate has been ignored.

Our Dye 3 result can also be compared with measurements of ^{36}Cl in rain and groundwater from the mid-latitudes. The ^{36}Cl deposition rate measured¹ in Long Island, New York rain collected during the bomb peak (1957–60), when corrected¹⁷ for the precipitation rate, is 2.3 atoms $\text{cm}^{-2} \text{s}^{-1}$. A recent measurement of ^{36}Cl in groundwater from Ontario, Canada¹⁷ gave a peak value of 4.8 atoms $\text{cm}^{-2} \text{s}^{-1}$. Our result for Dye 3, 0.5 atoms $\text{cm}^{-2} \text{s}^{-1}$, is a factor of 5–10 lower than these mid-latitude values. This is probably due to a combination of the latitude dependence of the stratospheric fallout and the additional contribution from direct tropospheric fallout from the Northern Hemisphere tests at lower latitudes.

The predicted mean lifetime of ^{90}Sr in the stratosphere using the model of Peterson, which takes into account the altitude and latitude of injection, is about 2 yr. This value is somewhat less than our measured value of ~3 yr for ^{36}Cl . Although this discrepancy may not be significant (an exponential with a slope of 2 yr is almost within the error bars) possible explanations are (1) contributions from additional tests during the period 1960–70 of which we are unaware and (2) fractionation between Cl and Sr in the transport out of the stratosphere.

After the Limited Test Ban Treaty in 1963, there were several smaller atmospheric tests listed in Table 2. Because these tests were smaller, higher above the ocean, and in the Southern Hemisphere their contribution to the total anthropogenic ^{36}Cl is small and hard to estimate accurately. However, they may be the cause of the small peaks seen in the data in the early 1970s.

It may be possible to use variations in the production of cosmogenic ^{36}Cl to study past solar activity as seen through the Sun's modulation of the galactic cosmic ray flux. The atmospheric residence time for ^{36}Cl is much less than that for ^{14}C so that time resolution should be better and amplitude variations should be larger. Our upper limit for pre-bomb ^{36}Cl fallout is 8×10^{-3} atoms $\text{cm}^{-2} \text{s}^{-1}$ which is consistent with predictions^{18,19} of $1\text{--}3 \times 10^{-3}$ atoms $\text{cm}^{-2} \text{s}^{-1}$. Further measurements with older ice are planned.

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Thermonuclear ^{36}Cl pulse in natural water

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Radionuclides produced by atmospheric testing of nuclear weapons have been used as environmental tracers for the past 30 years. ^{185}W and ^{90}Sr have proved valuable in monitoring atmospheric circulation¹, ^{14}C has been applied to oceanic mixing², and ^3H has been routinely used as a tracer in the hydrologic system. Tandem accelerator mass spectrometry⁴ has made possible measurements of several long-lived radionuclides that were previously virtually undetectable. One of the potentially most valuable of these for studying the movement and mixing of groundwater and surface water is ^{36}Cl . ^{36}Cl is produced continuously in the terrestrial environment in very small amounts by spallation of argon by cosmic radiation and by the capture of naturally produced neutrons by ^{35}Cl . It was also produced in much larger amounts by neutron activation of seawater and released into the environment during atmospheric

thermonuclear tests. This ^{36}Cl pulse has many potential applications as a tracer in natural water systems. Here, numerical modelling and analyses of water samples indicate that in the mid-latitudes the fallout peak was 3 orders of magnitude above the natural background, and that the period of enhanced ^{36}Cl fallout was 1953 to about 1964.

Chloride is widely used as a natural and artificial tracer in studies of freshwater systems. In most conditions, it is negligibly sorbed on solid particles, which greatly enhances its potential value. However, the timing and size of the ^{36}Cl fallout pulse, which have not been reported previously, have to be known to make best use of the bomb ^{36}Cl as a tracer.

^{36}Cl differs from many other bomb-produced tracers in that it is neither a product of direct fission nor of fusion. Virtually all bomb ^{36}Cl originates from thermal neutron activation of ^{35}Cl . Sufficient chloride to produce measurable global ^{36}Cl

fallout is present only when nuclear explosions are detonated near seawater. Thus only reported explosions on barges or small islands, of sufficient yield to penetrate the stratosphere, have been used as input to our model. Explosion data were taken from refs 5, 6. Neutron activation production in seawater has been calculated by Dyrssen and Nyman⁷. Cloud heights for some tests are published by Glasstone⁸; if not available, they were estimated according to Peterson⁹.

The fallout from these stratospheric injections was simulated by means of the HASL atmospheric box model of Krey and Krajewski¹⁰, modified to simulate global, rather than hemispheric, fallout. The model distinguishes between the stratosphere above 21 km, the stratosphere below 21 km, and the troposphere. The fallout from one to the next is considered to be a first-order rate process, with rate constants derived from ^{109}Cd and ^{90}Sr fallout data¹⁰.

Table 1 ^{36}Cl fallout rates calculated from ^{36}Cl concentrations in modern water

Location	Date collected	Precipitation evapotranspiration (mm)	Measured concentration (atoms $^{36}\text{Cl l}^{-1}$) ($\times 10^{-7}$)	Calculated fallout (atoms $^{36}\text{Cl m}^{-2} \text{ s}^{-1}$)
Pre-1953 water				
(Before nuclear testing)				
Arizona ⁴		300/98%		
Tucson City Well B8	January 1979		11.7	23
Tucson City Well C13	January 1979		9.6	18
Tucson City Well A31	January 1979		6.6	12
Monkey Springs	July 1979		8.1	15
Benson Well	July 1979		7.4	14
St David Well	July 1979		6.5	12
		Mean	8.4	16
Madrid Basin Wells		400/98%		
535-7a			7.1	18
535-7b			8.3	22
535-5a			5.6	15
		Mean	7.0	18
Texas Well		660/98%		
AL-68-59-504	December 1979		2.9	12
Atmospheric nuclear testing period (1953-64)				
New York (Long Island) ¹¹		1150/0%		
Rainfall	August 1957		77	28,000
	September 1957		183	67,500
	April 1959		24	8,900
	May 1959		1.02	370
	February 1960		26.3	9,600
		Mean	62	23,000
Shallow Well	August 1957		64	23,500
Post-1964 water				
Arizona ⁴		300/98%		
San Pedro River	July 1979		9	17
Arizona (Tucson)		300/0%		
Rainfall	February 1982		0.26	25
Rainfall	March 1982		0.31	29
California (La Jolla) ¹⁹		250/0%		
Rainfall	March 1979		0.2	16
Mixed samples				
Arizona		300/98%		
Campbell Ave Well	January 1979		158	310
Texas Well		660/98%		
AL-61-58-803	December 1979		20	85
New York				
Lake Ronkonkoma ¹¹	September 1960	1,150/50%	26.8	4,600
Lake Ontario ⁴	May 1978	750/50%	9.5	1,100
	June 1978	750/50%	5.4	640
Vermont ¹¹		1,150/50%		
Stream	September 1957		26.8	4,600
Nevada ²⁰		380/98%		
Truckee River	August 1958		167.5	420

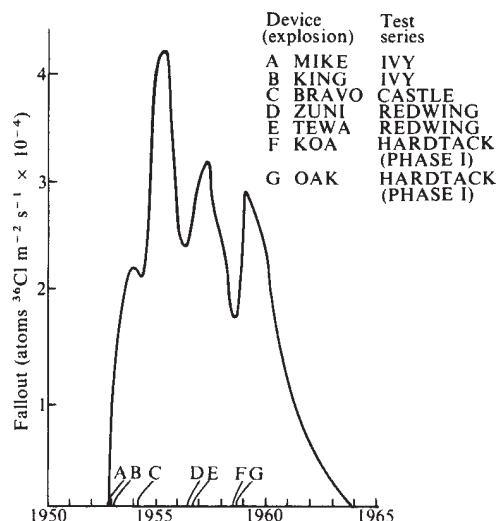


Fig. 1 Fallout of thermonuclear ^{36}Cl predicted by the atmospheric box model.

The model was calibrated using ^{36}Cl concentrations in rainfall on Long Island reported by Schaeffer *et al.*¹¹. In five samples of rainwater consisting of composites collected over 2–4 week periods between 1957 and 1960, the mean fallout rate was 2.3×10^4 atoms $^{36}\text{Cl} \text{ m}^{-2} \text{ s}^{-1}$ (Table 1). The data show a wide scatter which is typical of radionuclide concentrations in rainfall. The fallout at Long Island calculated from these data was converted to global fallout according to the latitude dependence published by Peterson⁹. The model results using this calibration are illustrated in Fig. 1.

Available data on ^{36}Cl concentrations in modern water are summarized in Table 1. The water samples may be divided into four categories: (1) water collected before nuclear testing, (2) concurrent with testing nuclear devices in and near ocean water, (3) postdating the testing and (4) mixtures from different periods.

The 'pre-bomb' well waters from Spain, Arizona, and Texas have been confirmed as such by ^3H and ^{14}C dating but are young enough so that measurable ^{36}Cl decay would not have occurred. ^{36}Cl -to-chloride ratios of these samples conform to the geographical distribution of naturally produced ^{36}Cl fallout predicted by Bentley and Davis¹².

The only undiluted sample of ground water from the period of atmospheric nuclear testing is from a shallow Long Island well reported by Schaeffer *et al.*¹¹. The ^{36}Cl concentration of this sample is close to the average for rainfall from the same period.

The San Pedro River sample is probably completely post-bomb, inasmuch as the river is largely ephemeral. The La Jolla and Tucson rainfall samples indicate that the ^{36}Cl content of present-day precipitation has returned to approximate pre-bomb levels. The remainder of the analyses are from hydrological systems which tend to mix water over long time periods, such as lakes, perennial rivers, and recharge areas of aquifers.

Although the data in Table 1 are reasonable in view of the fallout predictions, they yield little information concerning shape and magnitude of the pulse. To obtain more information, we sampled water from the Borden Canadian Forces Base landfill site in Ontario, Canada in November, 1981. The hydrogeology and geochemistry of the site, including the locations we sampled, have been thoroughly investigated^{13–15}. The tritium distribution has been investigated by Egboka *et al.*¹⁶. We sampled vertical profiles through the 'plume' of recent recharge delineated in the cited studies. The ^{36}Cl concentrations of samples collected from nested piezometers T5-A and B and T6-A and B are given in Table 2 and the data from T5 are illustrated in Fig. 2. Piezometer T5 penetrates the centre of the

Table 2 ^{36}Cl in groundwater from the Borden Canadian landfill site

Piezometer	Depth to sampling (m)	Atoms $^{36}\text{Cl} \text{ l}^{-1}$ ($\times 10^{-8}$)
T5A-3	8.84	1.69 ± 0.17
T5A-5	10.97	4.86 ± 0.38
T5A-6	12.03	7.45 ± 1.20
T5A-8	14.14	10.85 ± 0.76
T5A-9	15.24	9.26 ± 0.61
T5B-2	17.00	17.92 ± 1.13
T5B-3	18.29	15.86 ± 1.05
T5B-5	20.00	0.77 ± 0.12
T5B-9	24.00	2.17 ± 0.14
T6A-4	10.97	2.90 ± 0.23
T6A-6	14.02	2.71 ± 0.14
T6A-8	16.76	7.14 ± 0.94
T6B-2	19.51	0.35 ± 0.04

plume, but T6, on the edge of the plume, is not expected to give a representative sampling of the fallout.

Owing to uncertainties in the flow patterns, the sample depth cannot be definitely correlated with the date of recharge. However, the shape of the pulse is similar to that predicted by the fallout model. Furthermore, the maximum ^{36}Cl concentration is three orders of magnitude above the natural concentration estimated from the precipitation, evaporation, and chloride data of McFarlane *et al.*¹³ and Egboka¹⁷ and the geographical distribution of $^{36}\text{Cl}/\text{Cl}$ in pre-1953 water estimated by Bentley and Davis¹². The magnitude of this peak agrees with that of the fallout peak predicted on the basis of Krey and Krajewski's model and calibrated with Schaeffer's data. The maximum fallout rate calculated using the figures from the Borden site is 4.8×10^4 atoms $^{36}\text{Cl} \text{ m}^{-2} \text{ s}^{-1}$, compared with the predicted maximum of 4.2×10^4 atoms $^{36}\text{Cl} \text{ m}^{-2} \text{ s}^{-1}$. The total amount of ^{36}Cl in the vertical profile is 5.75×10^{12} atoms m^{-2} , whereas the total fallout predicted by the models is 7.1×10^{12} atoms m^{-2} .

Some preliminary data have also been obtained from an ice core sample at the Dye 3 site in Greenland¹⁸. The timing and

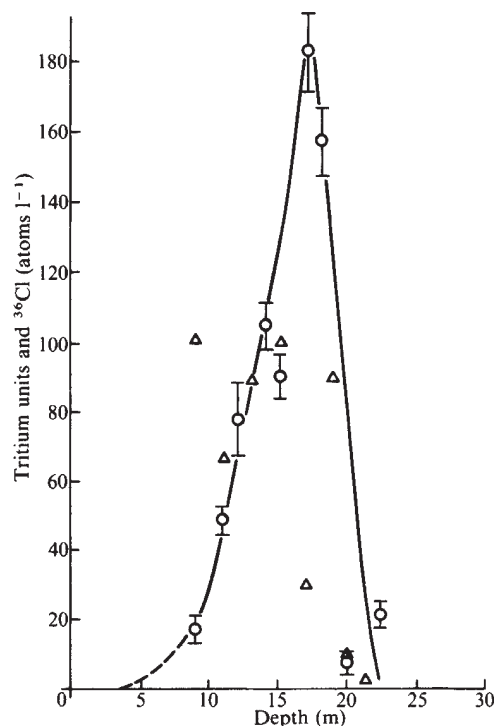


Fig. 2 ^{36}Cl ($\times 10^7$, $\circ$) tritium 16 (Δ , error ± 10 tritium units) concentrations in ground water from the Borden Canadian Forces Base landfill in Ontario, Canada. Data for T-5 nested piezometers are plotted.

the general shape of the peak are similar to that shown in Fig. 1, but the maximum concentration is only 80 times the minimum. This contrasts with the 900-fold difference shown in data of Table 1. The contrast could be due to the fact that minimum values measured in the ice may be above background or due to the latitudinal dependence of the fallout. For example, fallout of ^{185}W from the Hardtack series of explosions in the Pacific (which were major ^{36}Cl -producing tests) was more than an order of magnitude greater at 40°N than at 64°N (ref. 1).

We conclude that data obtained thus far are consistent with the ^{36}Cl fallout predictions and that the pulse lasted from 1953 to 1964. In contrast with ^{14}C and tritium, ^{36}Cl fallout rates have returned to levels existing before thermonuclear testing. In the mid-latitudes, the bomb pulse peak height was about 1,000 times the natural background level.

^{36}Cl pulse characteristics appear to be well suited for use as an environmental tracer. The maximum fallout rate was large enough to be unequivocally distinguished from natural con-

centrations, even after substantial dilution with stable chloride from natural systems. ^{36}Cl is one of the few hydrophilic anthropogenic tracers which has returned to natural levels since the time of the maximum input. This characteristic should be particularly valuable in the study of hydraulic flow and dispersive mixing. Decay of radionuclides and dispersive mixing of ground water create decreases in concentration which are difficult to distinguish. The long half life of ^{36}Cl should eliminate such ambiguities in interpretation of the bomb pulse. We expect that in the near future bomb-produced ^{36}Cl will usefully supplement bomb tritium as an environmental tracer, and that, as the bomb-tritium pulse decays to background levels, ^{36}Cl may eventually replace it.

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The evolution of propagating rifts

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The magnetic survey carried out by Mason and Raff^{1,2} off the north-west coast of the US has been central to the development of many of the concepts of seafloor spreading and plate tectonics. Recently Hey and Wilson³ have again used this survey to illustrate Hey's suggestion⁴ that ridge segments sometimes increase their length by propagation. This process leads to a characteristic pattern of magnetic anomalies offset by discontinuities which Hey called pseudofaults because, unlike fracture zones, they (Fig. 1) were never plate boundaries. We demonstrate here how the geometry of the pseudofaults, magnetic anomalies and the failed rift can be worked out using velocity triangles and the concept of stability. This method was originally developed to understand the evolution of triple junctions between three plates⁵.

Hey's⁴ suggestion is illustrated in its simplest form in Fig. 1a. The plate boundary between plates A and B, moving without rotation, consists of two ridge segments normal to the slip vector between the plates and which spread symmetrically with half rate ν . The transform fault which joins them is not stationary in a frame of reference fixed to A or B, but moves in the direction shown with a constant velocity V . This behaviour causes rift 1 to increase in length, rift 3 to decrease, and generates a complicated pattern of magnetic anomalies. Though it is possible for ridges and trenches also to move at right angles to their strike, the effects of such motion are geometrically less interesting and are easily analysed. In the case of ridges such motion leads to asymmetric spreading, whereas with trenches it leads either to consumption of both plates or to generation of new plate, presumably by back arc spreading. Whether the real behaviour of a propagating rift will be as simple as that illustrated in Fig. 1a is not clear. Analogue experiments with wax⁶ suggest that the deformation between the rifts may be distributed, rather than confined to a

single boundary, and that rotation may also be important. Such possibilities are, however, ignored here, and the discussion of the evolution is restricted to the simple two plate problem.

Figure 1b shows the representation of Fig. 1a in velocity space. Since plates A and B both move rigidly they can be represented by two points separated by a distance 2ν . The mid point of the line joining these two points corresponds to one frame of reference in which the ridge axes do not move. Furthermore any frame corresponding to points on the line marked ab_1 , ab_3 passing through this mid point and drawn parallel to the axis also leaves the ridge axes stationary because they are straight. Similarly lines ma and mb correspond to frames in which the magnetic anomalies on plates A and B respectively are stationary. As the transform fault is moving with respect to both plates, it is stationary in any frame corresponding to a point on line ab_2 , parallel to the line joining A and B but a distance V from it. It is now straightforward to construct the geometry produced by the motions in Fig. 1a. Unlike the corresponding problem involving three plates, the two plate system cannot be unstable. The point where the lines ab_1 , ab_3 and ab_2 intersect, marked P, corresponds to the frame in which the plate boundary system in Fig. 1a is stationary. Hence all points on A will move in the direction PA in this frame, those on B in the direction PB. Hence the traces of the tips of ridges 1 and 3 will be straight lines passing through the ridge tips, parallel to directions PA and PB, shown as dotted lines in Fig. 1b. Hey and Wilson³ refer to the two lines passing through the tip of ridge 1 as pseudofaults and that through the tip of ridge 3 as a failed rift. The geometry of the magnetic anomalies is slightly more difficult to construct. Because the motion of the transform fault is continuous, the region between the pseudofault and the failed rift on plate B must contain continuous anomalies. These are being lengthened by new material added to B from A by the movement of the fault. The magnetic anomalies on plate A and the transform fault are both stationary where the lines marked ma and ab_2 intersect at the point M_a in Fig. 1b. All points on plate B, including the material added to B by the movement of the fault, must move in the direction of the line M_aB , which is therefore the strike of the magnetic anomalies formed by the transfer of material onto B. The pattern of magnetic anomalies can then be drawn. The resulting

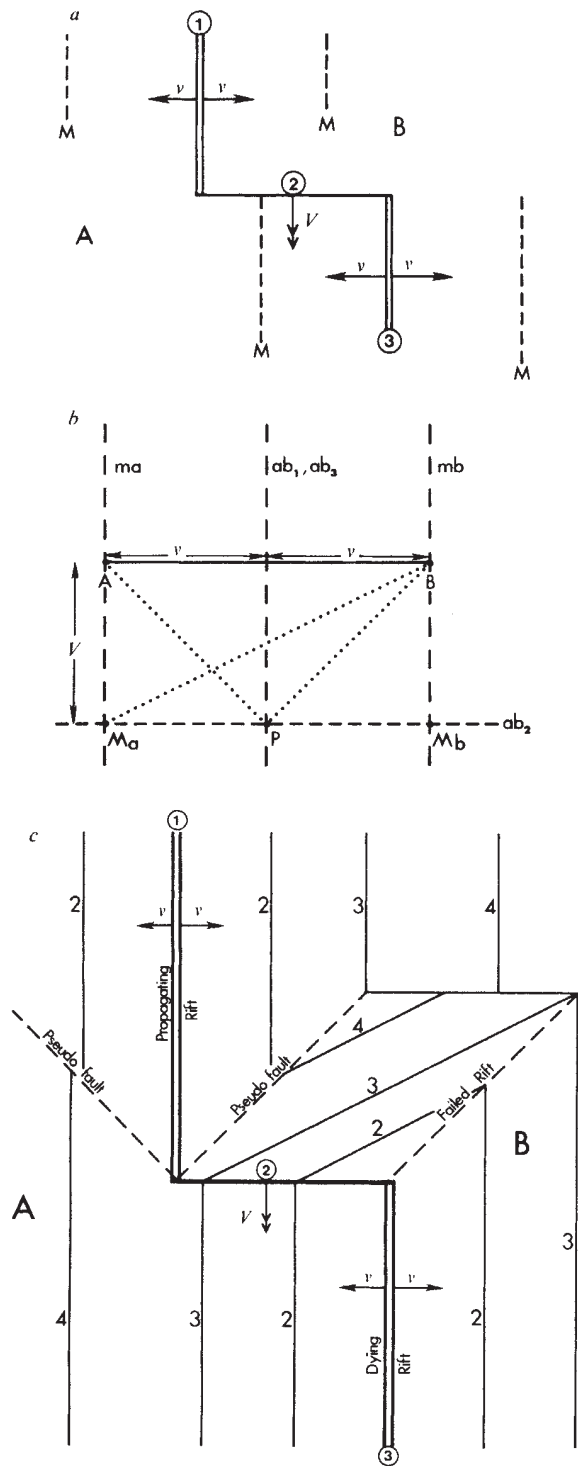


Fig. 1 *a*, Two plates A and B are separating with velocity $2v$ along a plate boundary consisting of two ridges joined by a transform fault. This fault moves with velocity V in the direction shown relative to both plates. *b*, The geometry in velocity space, with dashed lines representing frames in which features remain stationary. For instance any moving frame with a velocity on ma leaves the geometry of the magnetic anomaly M on A unchanged. The pattern of magnetic anomalies produced by these motions is shown in *c*.

arrangement agrees with that shown by Hey *et al.*⁷. Also shown in Fig. 1c is the relationship of the anomalies formed by the propagating rift to the anomalies older than anomaly 2 formed by the same rifts when they were not propagating.

Hey's original discussion was concerned with a more complicated geometry than that shown in Fig. 1a because one of the

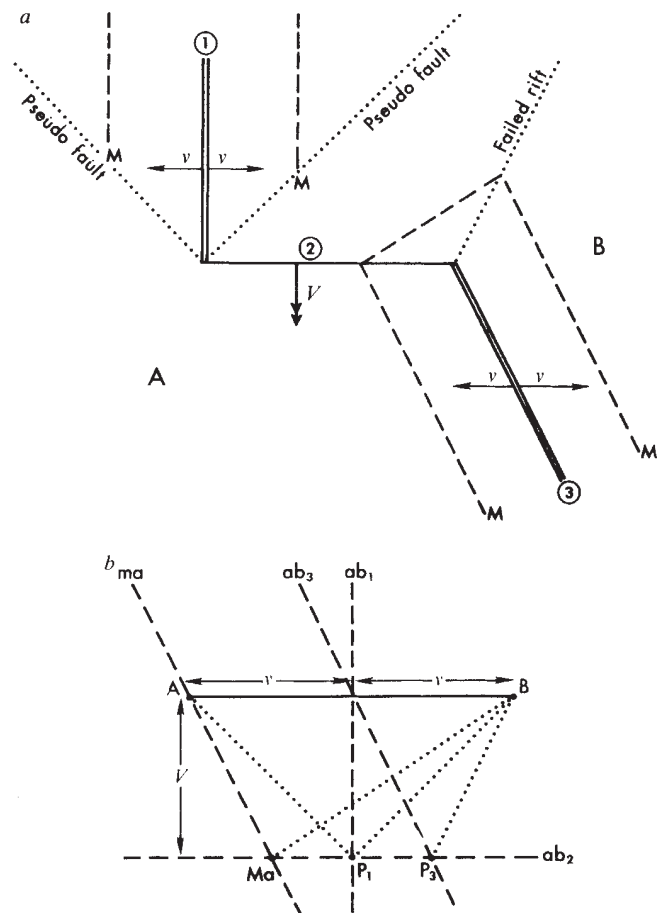


Fig. 2 *a*, As for Fig. 1, but with oblique spreading on one of the ridge segments. *b*, The geometry in velocity space.

ridges involved was spreading obliquely, though still symmetrically. In such conditions it is probable that the ridge which is not spreading obliquely will be the one which propagates, and this geometry is shown in Fig. 2. The evolution may be worked out in exactly the same way as before. Since the two ridges are not parallel, ab_1 and ab_3 intersect ab_2 in different places, P_1 and P_3 . Therefore there is no frame in which all three boundaries remain stationary. The tips of the two ridges are separating with a velocity given by the length of the line joining P_1 to P_3 , and the pseudofault on plate B is not parallel to the failed rift. Hence the geometry in Fig. 2a can be formed by propagation from a ridge axis with no transform fault, provided part of the ridge is spreading obliquely⁴. The strike of the magnetic anomalies produced by the transfer of material from A onto B is parallel to M_aB in Fig. 2b as before. Further complications such as asymmetric spreading can easily be included in these constructions.

Hey and his colleagues^{3,4,7} have convincingly argued that the magnetic anomalies off the coasts of Oregon and Washington are cut by pseudofaults. What is, however, less clear is whether the anomalies formed by the transfer of material from one plate to another by the movement of the transform fault can be recognized. Detailed surveys of areas where rifts may be propagating are required.

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Gabbroic rocks from the Mathematician Ridge failed rift

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Plutonic rocks from the ocean crust provide direct evidence on the petrological processes leading to its formation and evolution. The nature of such rocks is important for interpreting the origin of on-land ophiolites. So far, oceanic plutonic rocks have been mainly recovered from slow-spreading ridges and their associated fractures¹⁻¹³ and from trenches^{14,15}. Here we report the results of petrological studies of a suite of gabbros and related plutonic and volcanic rocks dredged from the Mathematician Ridge (MR), which was a fast-spreading ridge (43 mm yr⁻¹) until it was abandoned ~3.5 Myr ago¹⁶. Textures and mineral chemistry of the rocks together with structural considerations indicate that before abandonment the MR produced fractionated mid-ocean ridge basalt (MORB) and fractionated gabbros. The gradual decrease of spreading rate during abandonment resulted in morphological change, eruption of more primitive MORB and possibly tectonic emplacement of the older ferrogabbros. Alternatively, the latter could have been tectonically emplaced after abandonment.

The MR failed rift consists of a series of short (~50 km) *en echelon* troughs, offset by failed transform faults¹⁶; its present morphology (Fig. 1) is very similar to that of slow-spreading ridges like the Mid-Atlantic Ridge^{17,18} and the Mid-Cayman Rise¹⁹ although magnetic anomalies indicate that before abandonment it was a fast-spreading (43 mm yr⁻¹) ridge¹⁶. Two dredges of the upper portions of the trough walls (Fig. 1) recovered cumulate gabbro, isotropic gabbro, serpentinized peridotite, diabase, and MORB lavas. Many samples are extremely fresh but most are deformed and metamorphosed or brecciated.

Examination of 67 thin sections and microprobe mineral analyses of about 20 representative samples are summarized

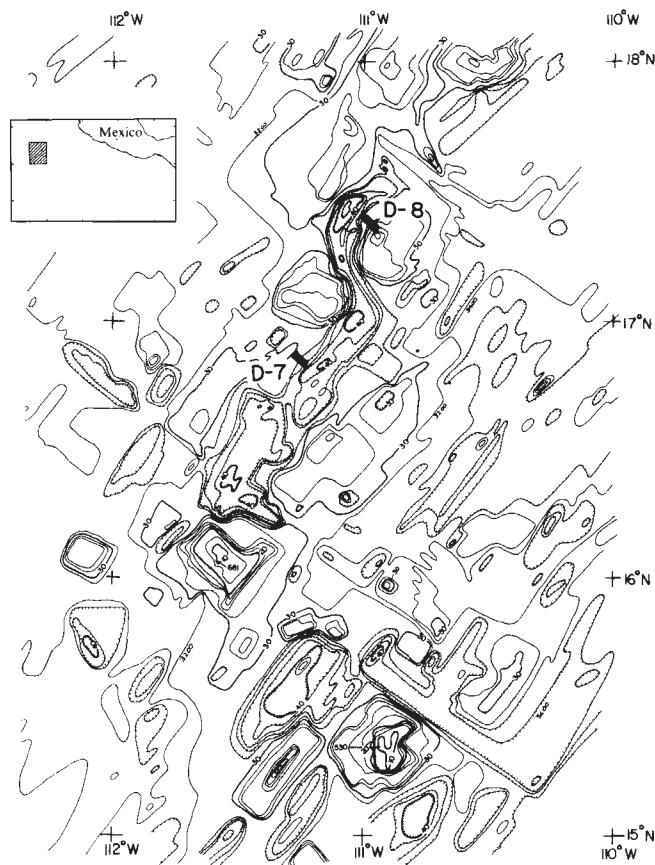


Fig. 1 Bathymetric map (contour interval 200 m) of part of the Mathematician Ridge. Locations of the two dredges that recovered plutonic rocks are shown as solid bars.

in Tables 1 and 2 and Fig. 2. The fresh troctolite and other cumulate gabbros have typical cumulate textures, including foliate plagioclase tablets and centimetre-scale mineral layering. Other fresh gabbros have isotropic texture and lack mineral layering. Secondary alteration of these fresh rocks consists only of weak uraltization of pyroxenes (<1%) and very rare

Table 1 Petrographic data for Mathematician Ridge plutonic rocks

Rock type	Troctolite	Cpx gabbro	2-PX Gabbro	Metagabbro	Amphibolite	Breccia
No. of samples	2	21	5	30	4	4
Mode for sample no.	8-7	8-14	8-16	7-1	7-19	8-25
Chemistry for sample no.	8-7	8-14	8-16	7-37	7-43	8-25
No. of points in point count	2,000	2,500	2,500	2,500	2,500	1,500
Olivine (ol)	23% (Fo ₇₃)					
Orthopyroxene (opx)*	1% (Wo ₀₂ En ₇₃ Fs ₂₅)		12% (Wo ₀₄ En ₄₆ Fs ₅₀)			
Clinopyroxene (cpx)	16% (Wo ₄₄ En ₄₄ Fs ₁₂)	22% (Wo ₄₄ En ₄₂ Fs ₁₄)	22% (Wo ₄₄ En ₄₁ Fs ₁₅)	11% (Wo ₄₆ En ₃₇ Fs ₁₇)		17% (Wo ₄₃ En ₃₇ Fs ₂₀)
Plagioclase (plag)†	60% (An ₇₂₋₆₀)	78% (An ₆₇₋₆₀)	66% (An ₆₀₋₅₁)	31% (An ₃₄₋₃₀)	2% (An ₀₂)	67% (An ₅₁₋₄₆)
Amphibole (amph)			<1%	34% (An ₃₄₋₃₀)	80% (An ₀₂)	15% (An ₅₁₋₄₆)
Clay				9%	9%	
Opakes	<1%	<1%	<1%	13%	9%	1%
Textures	Fresh cumulate plag laths (<6 mm); interstitial cpx and ol oikocrysts; opx rims on ol	Fresh, plag lath (<6 mm) mildly foliate, with cpx oikocrysts (<20 mm)	Cumulate plag (<7 mm) and opx (<4 mm) with interstitial cpx; slight uraltization	Myonitic, relict igneous textures, albite veins in plag	Decussate aggregate of hb; mild foliation of opakes; interstitial clay clots	Single- and multi-crystal clasts, subrounded, poorly sorted, cemented by clay-amph matrix
Deformation‡	A	ABC	ABC	CDEF	DF	BG

Accessories not tabulated (all <1%) are epidote, prehnite, quartz, sphene, apatite, analcime; observed exclusively in metagabbro, amphibolite, and breccia, or rarely in late veins through fresh gabbro. Note that as plag becomes more albitic, opx and cpx become more iron-enriched. Metagabbro has cpx similar to that in fresh gabbro, but plag more sodic than in gabbro. Amphibolite has only albite plag, and no cpx. Breccias are inferred to be brecciated gabbro that preserve primary plag and cpx compositions.

* Pyroxene compositions given in terms of molar components wollastonite (CaSiO₃), enstatite (MgSiO₃), and ferrosilite [(Fe, Mn) SiO₃].

† Plagioclase compositions give range of normal zoning of plagioclase laths. Discontinuous anorthite-rich rims, present in the fresh troctolite and gabbros, are not included.

‡ Deformation styles observed in this rock type: A, none except inclusion planes that appear to be healed fractures; B, bent pyroxene lamellae; C, stress-induced plagioclase twins and wavy extinction; D, shear bands, augen, porphyroblasts; E, 'mortar' texture; F, quartz-epidote fracture fills; G, brecciation.

Table 2 Representative microprobe analyses of pyroxene and amphibole

Mineral	Orthopyroxene	Clinopyroxene	Amphibole		Amphibole		
Rock no.	8-16	8-16	7-43		7-43	7-37	7-37
Analysis no.	(1)	(2)	(3)	1 σ	(4)	(5)	(6)
SiO ₂	52.92	51.38	43.75	0.76	52.24	44.22	45.28
TiO ₂	0.47	0.87	1.42	0.04	0.85	2.22	0.36
Al ₂ O ₃	1.17	2.31	11.84	0.47	4.33	8.76	8.11
Cr ₂ O ₃	0	0.02	ND	ND	ND	ND	ND
FeO ^T	19.25	9.03	12.25	0.53	12.14	18.27	24.51
MnO	0.41	0.24	0.13	0.04	0.16	0.26	0.18
NiO	0.04	0	ND	ND	ND	ND	ND
MgO	24.71	14.65	14.03	0.33	16.40	10.73	7.01
CaO	1.28	21.61	11.25	0.13	11.03	10.51	11.85
Na ₂ O	0.01	0.28	2.29	0.10	1.00	2.55	0.96
K ₂ O	0	0	0.07	0.01	0.02	0.10	0.07
Cl	ND	ND	0.25	0.03	0.11	0.50	0.12
F	ND	ND	0.01	0.03	0.04	0	0.01
Total	100.26	100.43	97.31		98.32	98.12	98.46
-O = Cl + F	0	0	0.06		0.04	0.11	0.03
Total	100.26	100.43	97.25		98.28	98.01	98.43
No. oxygen	6	6	23		23	23	23
Si	1.944	1.912	6.443		7.465	6.667	6.932
Ti	0.013	0.025	0.157		0.091	0.252	0.042
Al	0.051	0.101	2.055		0.729	1.557	1.464
Cr		0.001					
Fe	0.592	0.281	1.510		1.451	2.304	3.137
Mn	0.013	0.008	0.017		0.020	0.033	0.024
Ni	0.001						
Mg	1.353	0.813	3.080		3.495	2.412	1.602
Ca	0.050	0.862	1.777		1.690	1.697	1.945
Na	0.001	0.020	0.653		0.277	0.746	0.286
K			0.013		0.003	0.018	0.014
Cl			0.062		0.027	0.128	0.030
F			0.007		0.022		0.005
Total	4.018	4.023	15.774		15.270	15.814	15.481

Analyses: (1-2): electron microprobe analyses of representative orthopyroxene and clinopyroxene in a 2-PX gabbro (8-16). Other orthopyroxenes in 8-16 show some variation in iron content (up to ± 3 mol % Fs component). (3): Average and 1σ standard deviation of eight analyses of a 4-mm hornblende porphyroblast in 7-43 amphibolite. FeO^T represents all iron as Fe²⁺. (4): Core of a small (<1 mm) decussate-textured hornblende in 7-43. Such low-Al cores are zoned to rims that resemble the porphyroblast analysis (3). (5): Brown hornblende from 7-37 metagabbro. (6): 0.01 mm thick blue-green amphibole zone that flanks a tiny fracture through the brown hornblende in analysis (5) (7-37). ND, not determined.

serpentinite pseudomorphs of olivine crystals. However, the rocks contain evidence of anhydrous subsolidus reactions such as exsolution of pyroxene pairs whose compositions indicate equilibrium temperatures of 896-967 °C (ref. 20) and exsolution of Fe-Ti oxide rods and flakes from plagioclase. Furthermore, plagioclase (An₈₂₋₅₀) is often rimmed by discontinuous symplectic anorthite-augite growths or has simple anorthite (to An₉₂) rims (a feature also noted in other cumulates, see ref. 21). Similar textures may form by cotectic crystallization in interstices^{21,22}, but in Mathematician rocks, small inclusions of anorthite plus pyroxene (either low or high Ca types) plus Fe-Ti oxide occur within plagioclase. This suggests that the anorthite did not crystallize from a silicate liquid.

The olivine and pyroxenes in the fresh gabbros are fractionated (Fe-rich, see Table 1 and Fig. 2) relative to compositions of these minerals in many other oceanic gabbros. They are similar to the most fractionated ones in gabbro from the Mid-Cayman Rise¹⁹, and are more fractionated than practically all the same minerals from DSDP site 334 near the Mid-Atlantic Ridge⁴. The degree of differentiation of the Mathematician magma can be estimated using analysed olivines and Fe-Mg distribution coefficients. If $K_D = 0.30$ (ref. 23) then the range of magma Mg numbers ($Mg/(Mg + Fe^{2+})$) was 42-50, based on the range of measured olivine compositions Fo₇₁₋₇₇. Olivine compositions from other oceanic gabbros indicate that, if the olivines were cumulus phases and $K_D = 0.30$, the liquids had the following Mg numbers: Mid-Cayman Rise, 41-69 (ref. 19); DSDP site 334, 65-67 (ref. 4); Kane Fracture Zone, 45-67 (ref. 8); Mariana Trench, 49-63 (ref. 15). From the low Mg numbers and the absence of Mg-rich samples (Mg number > 50) in the Mathematician suite, it seems probable that these cumulate and isotropic gabbros formed while the MR was spreading rapidly²⁴⁻²⁶.

In fresh Mathematician gabbros, the mafic minerals generally become more Fe-rich as coexisting plagioclase cores become

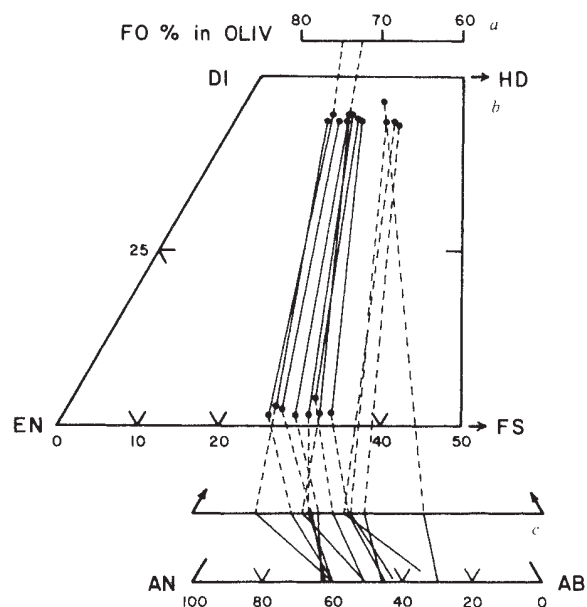


Fig. 2 Plot of electron microprobe analyses of minerals in plutonic rocks. a, Average olivine compositions in the two troctolite samples. b, Pyroxenes plotted in the conventional pyroxene quadrilateral (molar). The points are average compositions except for two (8-22 and 8-15) which are the most magnesian pairs of a small but detectable (6 mol % Fs component in cpx) intrasample variation. c, Molar plagioclase compositions in the Ab-An-Or ternary. Upper ternary represents the most calcic core in the sample, and lower ternary represents the most sodic rim. Discontinuous anorthitic rims (see text) are not plotted. Dashed lines connect analyses from the same sample.

more albitic (Table 1, Fig. 2). This suggests that the gabbros originated from multiply-saturated basaltic liquids undergoing slow cooling and fractional crystallization with some crystal settling contributing to the formation of cumulate gabbros.

Most gabbros from the MR are deformed and the extent of deformation is generally correlated with the extent of alteration/metamorphism of primary phases, except in brecciated rocks. These were apparently brecciated by brittle fracturing at low temperature, and contain chiefly clays as secondary minerals. In contrast, most deformed gabbros contain higher temperature metamorphic assemblages (see Tables 1 and 2). A continuous gradation from fresh gabbro to completely amphibolitized gabbro occurs among MR samples. This transformation is characterized by: (1) increase in the abundances of Cl-bearing amphibole (Table 2) at the expense of pyroxenes, and (2) concomitant decrease in the anorthite component in plagioclase: in completely amphibolitized gabbros, the scant plagioclase is albite, which may be retrograde. Additional evidence for retrograde metamorphism in the amphibolitized gabbros includes the presence of epidote (for example, Ps_{15-25} in sample 8-24; Ps_{14} in 8-17), prehnite ($Fe_2O_3 < 0.8$ wt% in samples 8-1, 8-18), albite veins, tremolite ($Trem_{66}Act_{34}$ in sample 8-16, $Trem_{62}$ in 8-24), chlorite, analcime, and quartz and quartz + Ep veins that cut foliation. The quartz veins contain polyphase fluid inclusions which sometimes contain a salt crystal. These inclusions and the Cl content of amphiboles indicate that late hydrothermal fluids were quite saline^{11,27}.

The serpentinite (82% talc/serpentine, 2% spinel, 16% clay and amphibole) contains Al-rich Cr-spinels [$Cr/Cr + Al = 0.4$; $Fe^{3+}/Fe^{3+} + Al + Cr = 0.01$; $Mg/Mg + Fe^{2+} = 0.64-0.70$] that have very rare inclusions of olivine (Fe_{91}). The spinels form coarse amoeboid grains, similar to a texture inferred by Dick²⁸ to occur characteristically in peridotite that has been partially melted. By contrast, cumulate spinel grown from a melt is normally euhedral²⁸. Therefore, textural evidence, together with the Al-content of the Cr-spinel, suggests that the serpentinitized peridotite represents partially-melted oceanic upper mantle material. Altered diabase recovered in dredge 8 contains secondary albite but relict clinopyroxene ($En_{51}Fs_{11}$) and is relatively unfractionated with $TiO_2 = 1.05$ wt%, $Cr = 370$ p.p.m., $La = 2.68$ p.p.m. and $(La/Sm)_N = 0.58$ (ref. 29 and work in preparation). Most of the MORB lavas recovered are also relatively unfractionated. Those from dredge 7 have Mg number = 74, $MgO = \sim 10$ wt%, $TiO_2 = 0.72$ wt%, $Cr = 500$ p.p.m., $Ni = \sim 200$ p.p.m., $La = 1.0$ p.p.m. and $(La/Sm)_N = 0.35$, while the most fractionated one, a MORB lava from dredge 8 has Mg number = 65, $TiO_2 = 1.28$ wt%, $Cr = 270$ p.p.m.

Plutonic rocks recovered in the MR are broadly similar to other oceanic gabbros, but they comprise a relatively fractionated suite. Detailed differences such as a lack of cumulus spinel, which is present in DSDP site 334 cumulates⁴, will be considered elsewhere. The suite of volcanic and plutonic rocks from the MR is similar to that found in ophiolites³⁰. However, because both dredges in MR troughs contained all rock types, we have no constraints on stratigraphical thicknesses, or even whether an orderly stratigraphy is exposed. If there is an orderly stratigraphy, then oceanic crust at the MR is very thin (<600 m, from Fig. 1). Otherwise, processes such as tectonic disruption or diapiric rise of serpentinite may have occurred. The chemical contrast between unfractionated MORB lavas and diabase and the relatively fractionated gabbros might suggest that these rocks are in tectonic contact, and gabbro breccias that formed at low temperature may have resulted from such tectonism. The timing of the presumed tectonic activity is poorly constrained, but some could have postdated the cessation of spreading and the inferred morphological changes associated with gradually decreasing spreading rate of the MR. We favour the notion that fractionated gabbros originated while the MR was spreading rapidly, whereas less fractionated MORB lavas were produced during the transition of the MR to its present slow-spreading morphology, because this is consistent with

petrological and geophysical data for presently active slow and fast-spreading ridges²⁴⁻²⁶. Choosing between this hypothesis and the possibilities discussed above will require further detailed studies of the MR and the stratigraphical/structural relationships of the rocks exposed there.

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Why is Mrs Thatcher interrupted so often?

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If a conversation is to proceed smoothly, the participants have to take turns to speak. Studies of conversation have shown that there are signals which speakers give to inform listeners that they are willing to hand over the conversational turn¹⁻⁴. Some of these signals are part of the text (for example, completion of syntactic segments), some are non-verbal (such as completion of a gesture), but most are carried by the pitch, timing and intensity pattern of the speech; for example, both pitch and loudness tend to drop particularly low at the end of a speaker's turn. When one speaker interrupts another, the two can be said to be disputing who has the turn. Interruptions can occur because one participant tries to dominate or disrupt the conversation. But it could also be the case that mistakes occur in the way these subtle turn-yielding signals are transmitted and received. We demonstrate here that many interruptions in an interview with Mrs Margaret Thatcher, the British Prime Minister, occur at points where independent judges agree that her turn appears to have finished. It is suggested that she is unconsciously displaying turn-yielding cues at certain inappropriate points. The turn-yielding cues responsible are identified.

Mrs Thatcher's interviews display a distinctive pattern—she is typically interrupted more frequently than other senior politicians, and she is interrupted more often by interviewers than she herself interrupts⁵. This pattern is consistent across a wide variety of interviewers (including Denis Tuohy, Brian Walden and Llew Gardner) and across a wide variety of topics⁶. Butting-in interruptions are particularly common in her interviews. These occur where her interviewer interrupts but fails to get the floor, as in the following example where Denis Tuohy (D.T.) tries to take the floor from Mrs Thatcher (M.T.) (*TV Eye*, April 1979).

M.T.: ... if you've got the money in your pocket you can choose/whether you spend it on things which attract Value Added Tax/or not

D.T.: (You s-

M.T.: (and the main necessities don't

D.T.: You say a little on Value Added Tax

where / indicates unfilled pause ≥ 200 ms

(word 1
(word 2 indicates simultaneous speech

A series of experiments was designed to test the hypothesis that Mrs Thatcher is often interrupted because she displays turn-yielding cues at points where she has not completed her turn. Forty extracts were selected from the Denis Tuohy *TV Eye* interview (ITV, April 1979): 10 were turn-final (that is, utterances at the end of a turn immediately preceding a smooth speaker-switch), 20 were turn-medial (utterances from within a turn), and the remaining 10 were turn-disputed (utterances immediately preceding an interruption by Tuohy). These extracts all contained at least one sentence. The extracts were presented to subjects who had to judge whether Mrs Thatcher's turn was complete or not, in a forced choice procedure. The extracts were presented on video to 79 subjects, on audio only

Table 1 Results of completion-judgment experiments to assess the reasons Mrs Thatcher is interrupted so often

	Video	Mode of presentation		Typescript
		Vision only	Audio only	
Turn-final	83.530	76.430	62.230	63.500
Turn-disputed	40.120	38.570	55.860	50.500
Turn-medial	23.045	18.570	32.405	58.250

Correlations between presentation modes (% completion judgements for each utterance)

Vision only	Audio only	Typescript	
0.898	0.676	0.083	Vision
	0.586	0.000	Vision only
		-0.080	Audio only

to 29 subjects (in the audio presentation, only the final tone group of each utterance was presented, with two exceptions in which the last two tone groups were played because the final tone group comprised only one word), on vision only to 14 subjects, and typescripts of the extracts were presented to 20 subjects. There were different numbers of subjects in conditions because the extracts were presented in university classes. All subjects were students at the universities of Sheffield or Sussex. For each of the 40 extracts in each of the 4 modes of presentation, the percentages of completion judgements were calculated (see Table 1).

An analysis of variance (ANOVAR) revealed that both 'type of utterance' ($F = 23.265$; $P < 0.0001$) and 'mode of presentation' ($F = 8.282$, $P < 0.0001$) significantly affected judgements of completion. There was also a significant interaction effect between type of utterance and mode of presentation ($F = 7.692$; $P < 0.001$). As predicted, turn-final utterances produced the

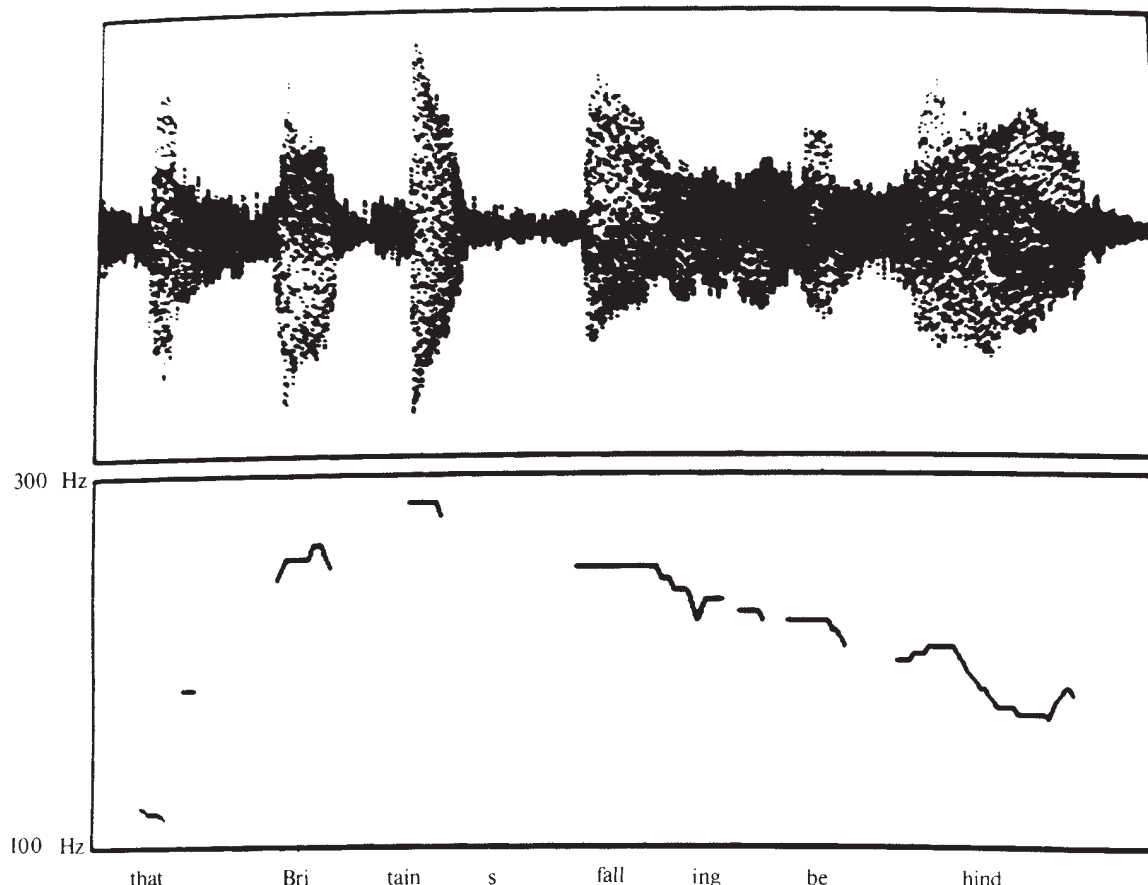


Fig. 1 Amplitude trace and pitch contour of turn-medial utterance, showing slow fall in utterance pitch.

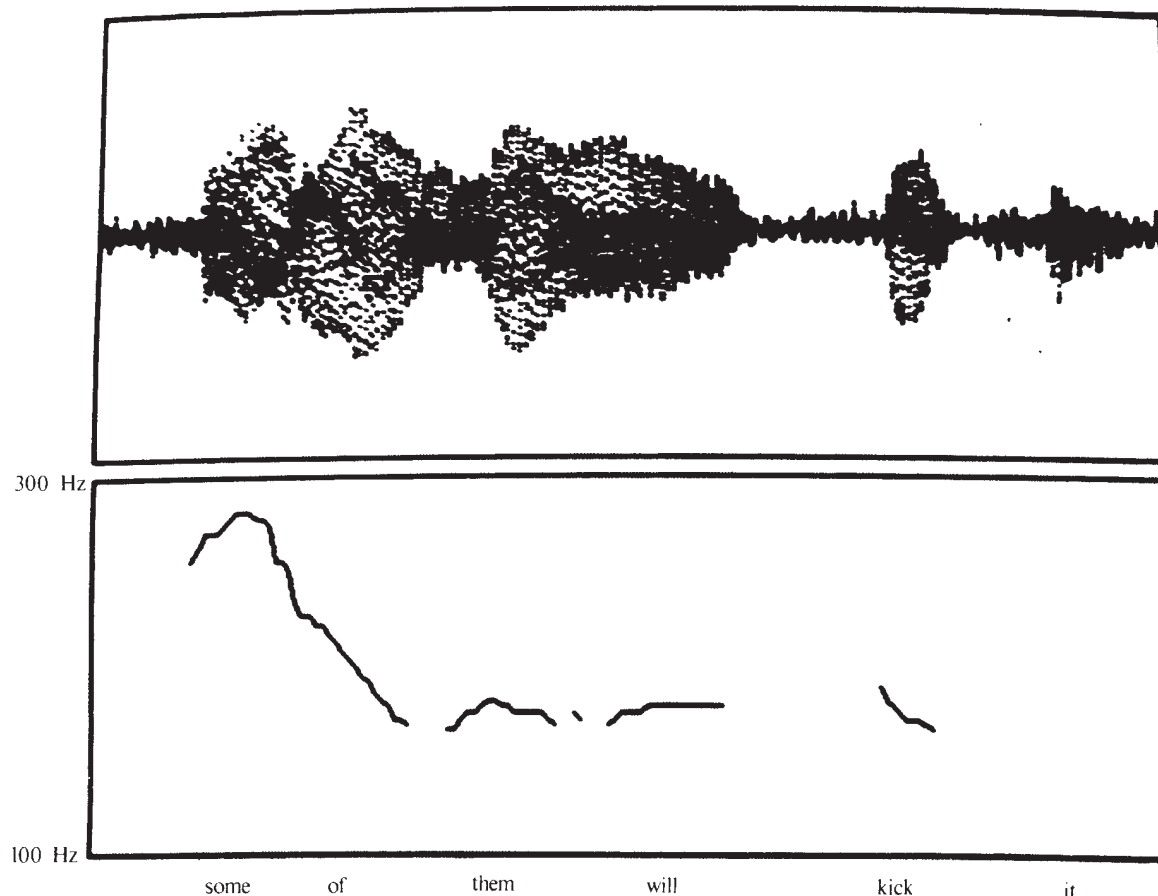


Fig. 2 Amplitude trace and pitch contour of turn-final utterance, showing fast fall in utterance pitch.

most completion judgements, and the turn-medial the lowest, with the disputed turns falling somewhere in between these two. One-factor ANOVAs revealed that 'type of utterance' had a significant effect in the case of the video presentation ($F = 34.791$, $P < 0.0001$), audio presentation ($F = 5.129$, $P < 0.02$) and vision-only presentation ($F = 25.486$, $P < 0.0001$). With the typescript, however, there was no significant effect ($F = 1.185$, not significant). (Note that significant effects did emerge in conditions with smaller numbers of subjects than the typescript condition, thus in fact demonstrating the robustness of the effects.) A comparison of means, using the Tukey HSD procedure (see ref. 12, p. 87) revealed that judged completion scores were significantly higher for turn-disputed utterances than for turn-medial utterances for the video presentation ($F = 5.53$, $P < 0.05$), the audio presentation ($F = 5.15$, $P < 0.05$) and the vision-only presentation ($F = 6.09$, $P < 0.05$).

Tests for correlation revealed significant positive correlations (all at the 0.001 level or beyond) among the video, vision-only and audio-only conditions for proportion of completion judgements for each utterance. The typescript condition judgements, however, did not correlate significantly with any of the other conditions (see Table 1). Thus, although, for example, the means for the turn-final utterances in the audio and typescript conditions are similar, the individual utterance ratings which comprise the means are obviously quite different.

Thus the basic hypothesis was supported—those utterances immediately preceding an interruption by the interviewer were judged to be complete more often than turn-medial utterances and this was not on the basis of syntactic or semantic content (present in the typescript) because, of course, no significant effect emerged in the case of the typescript, but on the basis of information carried in the audio channel and in the visual channel of the interaction, as models of turn-taking such as that of Duncan² would predict. Subsequent analyses sought to

determine exactly which signals were giving rise to subjects' perceptions of these utterances as complete.

First, the 40 extracts from the Denis Tuohy interview with Mrs Thatcher were digitized and coded into LPC (linear predictive coding) parameters, and a value for the fundamental frequency of each sample was extracted. All 40 utterances terminated in a pitch fall with one exception, a question which ended in a rise; this exception was excluded from further auditory analysis. The value of the fundamental frequency in cycles per second was determined for the highest and lowest points (peak and trough) of each terminal fall. The mean values for the three types of utterance are displayed in the first two columns of Table 2. Column 3 shows the mean pitch range covered by the fall (that is, the difference between columns 1 and 2), and column 4 gives the mean time in milliseconds over which the fall was realized.

ANOVAs on these four measures showed that on two of them, the three conditions differed significantly: (1) The trough of the fall; the difference between conditions was significant ($F = 5.75$, $P < 0.01$), and Scheffé *post-hoc* comparisons showed that the turn-final utterances ended significantly lower than the other two groups, which did not differ significantly from one another.

(2) The timespan over which the terminal fall was realized; the difference between conditions was significant ($F = 4.61$, $P < 0.02$), and Scheffé *post-hoc* comparisons showed that this effect was due to the turn-medial falls being slower than either of the other two types of utterance, which did not differ significantly (see Figs 1, 2). There was no significant difference between the three types of utterance on either the height of the pitch peak or the range of the fall.

This analysis suggests that the turn-disputed utterances may, in fact, have conflicting cues: a fast fall like the turn-final utterances, but a fall which does not descend very low, like the

Table 2 Mean peak and trough values for three types of utterance

	Peak	Trough	Δ	Span
Turn-final	238	141	97	693
Turn-disputed	263	167	96	463
Turn-medial	275	161	114	953

turn-medial utterances. This, in turn, suggests that while the speaker is actually giving a number of cues to the end of her turn, the cue which she considers paramount may be different from the cue which her interlocutor considers paramount. If she considered that her most decisive cue to the end of her turn was letting her voice drop to around 140 Hz instead of keeping it no lower than 160 Hz, whereas her interlocutor considered that her most decisive cue was a rapidly executed final fall rather than a slow fall, their respective decisions as to whether or not she had finished her turn would differ in precisely those cases which were disputed in the present sample of utterances.

One of the authors, trained in phonetic transcription, then transcribed the 40 utterances without knowledge of the position of the utterances in the speaker's turn. The transcriptions of the turn-final and turn-medial utterances were then separately inspected, and the prosodic and vocal quality features occurring in three or more of these utterances listed. Five 'final' features were found: pitch downstep (rapid drop) before the main fall (tonic); double falling contour; allegro portion before the tonic; whispery voice; and creaky voice. Three 'medial' features were found: allegro continuing through the tonic; pitch upstep before the final fall; and a non-falling sustained contour after the fall. (Note that because all our sample utterances ended in falls, we must have underestimated the complete range of cues when non-falling utterances are included. For example, many turn-medial utterances end in a fall-rise.)

The remaining utterances were then checked for the presence of these features, which were assumed to be characteristic of turn-final and turn-medial utterances, respectively. The five 'final' features occurred on average 2.2 times per turn-final utterance, but 0.2 times per turn-medial utterance. Turn-disputed utterances, with a mean of 1.6, were closer to the turn-final utterances than to the turn-medial utterances. The three 'medial' cues occurred on average 0.65 times per turn-medial utterance, but 0.10 times per turn-final utterance. Turn-disputed utterances again fell in between with a mean of 0.30.

The number of acoustic cues present in each utterance was then correlated with the results of the audio perception test, and the proportion of completion judgements for each utterance was found to have a significant positive correlation with the mean number of 'final' cues present in that utterance ($r = 0.46$, $P = 0.003$), but a significant negative correlation with the mean number of 'medial' cues ($r = -0.45$, $P = 0.004$). Thus, the listeners in this experiment seem to have been making use of these phonetic and vocal quality cues in making their forced choice judgements.

In terms of the visual channel, the main signal guiding completion judgements must have been eye gaze because, in a large proportion of instances, the camera angle was such that no information was available on other aspects of nonverbal behaviour such as gesture (gesture termination being an important turn-yielding signal, and gesture maintenance an important turn-holding signal, according to Duncan). Eye gaze has been shown in the past to be implicated in turn regulation⁷⁻⁹. Analysis of the video revealed that in 100% of the turn-final utterances, Mrs Thatcher was looking at her interviewer at the end of the utterance, compared with 55% of turn-medial utterances. Turn-disputed utterances were again intermediate with 80%.

We have argued that some interruptions in conversation may arise from the transmission of turn-yielding cues at inappropriate points rather than from any desire on the part of either participant to dominate or disrupt the conversation. It has often

been suggested that conversation can be regarded as a highly skilled performance^{10,11}. Successful manipulation of these very subtle turn-taking signals must certainly rank as one important aspect of this conversational skill.

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Is annual reproduction in deep-sea echinoderms a response to variability in their environment?

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The deep-sea environment has traditionally been considered as one of the least variable on the surface of the Earth¹⁻³. However, recent evidence⁴⁻⁶ suggests that there may be a seasonal fluctuation in the physicochemical environment, and there is further evidence⁷⁻¹¹ that annual reproductive periodicities may occur among populations of deep-sea invertebrates. We report here evidence for annual periodicities and considerable between-species synchrony in the reproductive cycles of five deep-sea echinoderms, spawning occurring in late winter and early spring. These species also show a similar mode of early development that suggests adaptation to a seasonally varying food supply.

One of the aims of a long-term time series sampling programme initiated by the Scottish Biological Association in the Rockall Trough (north-east Atlantic)^{12,13} is to test the hypothesis that annual periodicities may occur in deep-sea invertebrates. In 1975 a permanent station was established at 2,900 m depth (54°40' N; 12°16' W). In 1978 this station was supplemented by a second (station 'M') at a depth of 2,200 m nearer the base of the Hebridean Slope (57°18' N; 10°23' W). Samples of the benthic fauna have been taken from both stations, using either an epibenthic sled or an Agassiz trawl at approximately 4-monthly intervals to the present date.

Although a wide variety of deep-sea benthic invertebrates have been collected, echinoderm species dominate in terms of biomass. Seven echinoderm species (Table 1a) were collected in sufficient numbers to determine any seasonal reproductive cycles. A further seven species have been examined but were collected irregularly or in insufficient numbers to determine reproductive periodicity (Table 1b). In addition, echinoderm material is available to us from a number of other recently obtained samples from the Rockall Trough¹⁴, and fine-meshed rectangular midwater trawl (RMT 1; mesh diameter 0.33 mm) samples have been obtained over station 'M' to look for echinoderm larvae (Table 1c).

To investigate the reproductive cycle of each species, the gonads of each specimen were dissected out and processed to paraffin wax, sectioned and stained. In all species examined the sexes were separate and equal in numbers, and since gametogenesis is more easily determined in females, we use data on oocyte size frequencies (Fig. 1a-c) to study reproductive seasonality. The diameters of at least 100 oocytes per specimen were measured and from these data oocyte-size/frequency diagrams constructed (Fig. 1a-c).

Table 1 Reproductive biology of deep-sea echinoderms from the northern Rockall Trough

Species	Sample depth (m)	Total no. examined from time-series samples*		Maximum egg size (μm)	Annual reproductive cycle	Ref.
		Female	Male			
a, Species available for examination of seasonality						
<i>Ophiura ljunghman</i>	2,900	138	132	120	+	11
<i>Ophiomusium lymani</i>	2,200	180	132	420	Only larval recruitment	13
<i>Bathybiaster vexillifer</i>	2,200	67	51	900	—	15
<i>Plutonaster bifrons</i>	2,200	85	93	110	+	36
<i>Benthopecten simplex</i>	2,200	90	77	900	—	16
<i>Hymenaster membranaceus</i>	2,200	114	104	1,100	—	37
<i>Echinus affinis</i>	2,200	115	121	110	+	(Unpublished)
b, Other species examined						
<i>Dytaster insignis</i>	2,200	6	10	120	? +	36
<i>Psilaster andromeda</i>	2,200	10	12	900	—	36
<i>Pectinaster filholi</i>	2,200	34	31	850	—	16
<i>Pontaster tenuispinus</i>	590–1,050	17†	10†	850	—	16
<i>Pseudarchaster parellii</i>	2,200	7†	6†	900	—	17
<i>Paragonaster subtilis</i>	2,200	6†	15†	900	—	17
<i>Zoroaster fulgens</i>	2,200	34†	20†	900	—	(Unpublished)
c, Larvae of species collected by RMT1						
<i>Ophiacten gracilis</i>	1,000	—		110	+	25

* Immature, indeterminate, hermaphroditic and parasitized specimens are not included.

† Including additional material not from time series.

Among the 14 species of echinoderms (Table 1), two distinct reproductive strategies are evident. In nine of the species, a few, large-sized eggs ($\sim 1,000 \mu\text{m}$ diameter) are produced^{15–17}. This is usually interpreted as indicating direct development, omitting a larval stage^{18,19}. None of these species show any indication of seasonality in gametogenesis, eggs presumably being produced continuously throughout the year. The production of a few large eggs with considerable reserves of yolk is considered a result of evolutionary selection for increased efficiency of reproduction with minimal wastage of fertilized eggs, embryos or larvae before recruitment to the adult population.

An alternative reproductive strategy is seen in the remaining echinoderms examined (Table 1) and is particularly evident in *Ophiura ljunghmani*, *Plutonaster bifrons* and *Echinus affinis*. To these three species may be added *Ophiacten gracilis* and *Dytaster insignis*. All these species produce a large number of small ($\sim 100 \mu\text{m}$ diameter) eggs which develop as free-swimming larva and feed in the plankton^{18,19}. It is of great significance that these echinoderms show both between- and within-species synchrony in egg production (Fig. 1a–c). This is especially

evident in *O. ljunghmani* and *E. affinis*. In the former, gametogenesis is initiated in March/April, and gonad growth continues until maximum development is attained in November. Spent individuals are found in January and February each year. Oogenesis in *E. affinis* appears to start in the germinal epithelium of the ovary at the time of maximum development of the previous year's oogenic growth. The ripe oocytes are spawned in January/February and the newly developing oocytes within the germinal epithelium develop to reach maximum size in November/December, by which time the next cycle of oogenesis has been initiated.

In *P. bifrons* the annual oogenic cycle does not appear as discrete as that of *O. ljunghmani* and *E. affinis*. Gametogenesis can occur from January to at least May. There is a period of oocyte growth before spawning, which appears to occur from November to March.

The degree of variation in gonad development, both between samples and between individuals within samples, may be determined by the analysis of oocyte size/frequency data using a mixed model, nested-design, analysis of variance²⁰. The percentage of total variance, and the variance between individual

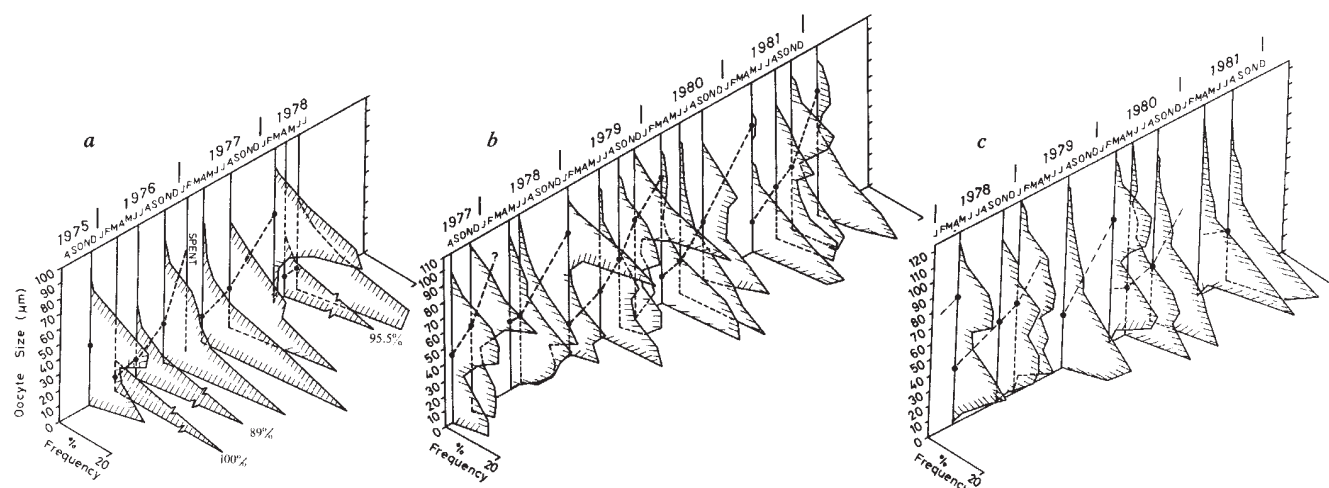


Fig. 1 The percentage frequency distribution (abscissa) for different oocyte sizes (ordinate) for each sample against time. *a*, *Ophiura ljunghmani*. *b*, *Echinus affinis*. *c*, *Plutonaster bifrons*. ● Represents mean oocyte diameter for each sample.

Table 2 The degree of gonad development in deep-sea and shallow-water echinoderm species

Species	Between samples*	σ_s^2 % Variance between samples	σ_1^2 % Variance between individuals	% Variance within individuals	j $100 \times \sigma_s / (\sigma_s + \sigma_1)$
<i>Echinus affinis</i>	21.2316 (11)	20.98	9.53	69.49	68.75
<i>Plutonaster bifrons</i>	9.4108 (12)	18.31	11.87	69.82	60.68
<i>Ophiura ljunmani</i>	22.1720 (13)	45.01	22.55	32.45	66.63
<i>Bathybiaster vexillifer</i>	3.3386 (9)	2.10	7.48	90.43	21.89
<i>Benthopecten simplex</i>	0.9261 (8)	0.0	21.82	78.18	0.0
<i>Hymenaster membranaceus</i>	3.5355 (10)	1.96	6.55	91.49	23.03
<i>Ophiomusium lymani</i>	4.6488 (16)	3.94	7.86	88.20	33.40
<i>Ophiura albida</i> †	18.0783 (20)	27.19	13.11	59.70	67.48
<i>Ophiura texturata</i> †	5.2900 (19)	11.24	20.64	68.12	35.25

* Value in parentheses indicates degree of freedom in numerator of *F*-ratio; degrees of freedom of denominator are effectively ∞ .

† *Ophiura albida* and *O. texturata* are shallow-water species included for comparison. j = % of variance between individual means explained by sample means.

mean oocyte size accounted for by the sample means is large (>60%) for *O. ljunmani*, *E. affinis* and *P. bifrons* (Table 2). These data are comparable with values for shallow-water species known to breed seasonally (Table 2)²¹. Highly significant variation in mean oocyte size occurs between samples within each species. By contrast, although there are significant differences in sample means for *Bathybiaster vexillifer*, *Hymenaster membranaceus* and *Ophiomusium lymani*, this accounts for a much smaller percentage of variance between samples (Table 2). In *Benthopecten simplex*, the sample means are not significantly different.

The values suggest that there is a distinct variation in mean oocyte size throughout the year in *E. affinis*, *O. ljunmani* and *P. bifrons* and confirms the observations of seasonal variation seen in the oocyte size/frequency figures.

These data indicate an annual reproductive periodicity that culminates in a spawnout of gametes in January/February each year. In addition to the evidence of seasonal reproductive cycles shown in gametogenic cycles of echinoderms, the occurrence of larvae has been followed in seasonal hauls using the RMT 1. The larval form, *Ophiopluteus ramosus*, was taken abundantly only in the spring (April to June) of each year. The spring-time occurrence of this larva has been noted for several years²²⁻²⁴. *Ophiopluteus ramosus* is now known as the larval form of *Ophiocten gracilis*²⁵. This brittle star has a distribution centred on 1,000 m, where we may conclude it also shows an annual reproductive cycle.

In addition to the reproductive data, the epibenthic-sledge time series has provided evidence of a late spring/summer-time recruitment of post-larvae of both *O. ljunmani* and *E. affinis*^{11,26}.

From these data we see that four species of deep-sea echinoderm, *O. ljunmani*, *O. gracilis*, *P. bifrons* and *E. affinis*, have highly synchronous reproductive cycles. We must infer that some exogenous factor(s) control synchrony of spawning and act as the ultimate selective factor determining reproductive seasonality²⁷.

Recent discoveries of seasonal periodicities in the physical environment and in sedimentary organic flux from the surface within the deep sea⁴⁻⁶ suggest environmental factors have a role in the control of reproductive seasonality in deep-sea populations. The reproductive cycle of benthic invertebrates must be controlled at several levels: (1) initiation, (2) gamete development, (3) spawning, and (4) the period of larval development. Factors controlling both initiation of gametogenesis and gamete development are difficult to define²⁸ even in shallow-water species, although energy available as food will be a significant controlling factor for vitellogenesis. In shallow water, spawning is known to be precipitated by changes in the physical environment such as seasonal temperature fluctuations. Although such changes have been considered imperceptible in the deep sea, recent long-term measurements of current velocity show oscillations of varying period from semidiurnal and diurnal

tidal components to low frequency signals, with seasonal maxima in late winter and minima in summer, in surface-generated kinetic energy^{6,29}. Although rapidly diminished with depth, the seasonal effects of such kinetic energy (that in the Rockall Trough perhaps are associated with travelling disturbances created by mixing of Atlantic and Norwegian Sea water²⁹) may be transmitted to abyssal depths well exceeding that of the 2,900 m deep Permanent Station⁶. Time-lapse photographs have shown that the behaviour of deep-sea fish may be influenced by semidiurnal tidal currents in the Bay of Biscay³⁰. However, the possible role of low-frequency physical periodicities as proximate cues in triggering spawning of ripe echinoderms remains speculative. Possibly, secondary effects such as periodic sediment resuspension above critical erosive current velocities at the benthic boundary³¹ are more important than direct effects of water motion on echinoderms lying on the seabed. However, chance, isolated triggering of spawning of an individual may be more rapidly transmitted throughout a ripe population by chemical signals in times of high water motion than in quieter periods.

Of considerably greater significance is the inference that in all the seasonally-reproducing deep-sea echinoderms, larvae are produced from February to June, coinciding with the spring-time maximum in phytoplankton production in this area of the north-east Atlantic³². We believe that there is a sufficiently rapid transfer of small organic particles resulting from surface production, to the deeper water where, together with its associated bacteria, it would be available as food for the free-floating planktotrophic larvae. There is considerable evidence that this transfer of surface production to deep water is both rapid³³⁻³⁵ and highly seasonal^{4,5}. Such a cycle in the availability of surface-derived food material would be expected to influence the evolutionary selection for annual reproduction; larvae produced outside the period of elevated food supply being unlikely to survive.

Although we believe that this hypothesis is valid for deep-sea echinoderms with annual reproductive cycles, we have yet to determine whether such factors determine the reproductive strategies of other deep-sea invertebrates.

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Analysis of the dual role of phytochrome in the photoinhibition of seed germination

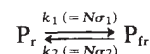
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Light can both promote and inhibit germination, even of seeds of the same species^{1–3}. Many seeds considered to be positively photoblastic are inhibited by prolonged irradiation with light of a wide variety of spectral qualities, including those, such as sunlight, that promote germination when given as a short exposure⁴. The promoting effect of light is mediated by the red/far-red reversible receptor pigment phytochrome operating in its P_r form. The nature of photoinhibition is much less clear, although it is possible that continuous excitation of phytochrome is involved⁵. It has been suggested that light could control photomorphogenesis in seedlings not only through the establishment of a particular amount of phytochrome in its active P_r form but also by determining the rate of 'cycling' between P_r and P_{fr} forms^{6,7}. Such effects of prolonged irradiation are both wavelength and fluence rate dependent. We show here that the photocontrol of seed germination in *Sinapis arvensis* L. involves a promoting reaction dependent on the proportion of phytochrome in the P_r form at photoequilibrium (ϕ) and an inhibiting reaction dependent on the rate of phytochrome interconversion or 'cycling' (H). Thus percentage germination can be quantitatively predicted from the spectral quality and the fluence rate of the seed's light environment.

The photoinhibition of seed germination under prolonged light does not show reciprocity (dependence on the product of fluence rate and exposure time), the length of the irradiation period being an important factor independently of total fluence. The action peak of the response occurs at about 710–720 nm in the few species studied, such as *Lactuca sativa* L.^{7,8} and *Poa pratensis* L.⁹. This is characteristic of the 'high irradiance reaction', most studied in seedling photomorphogenesis^{7,10,11}. However, in seed germination the high irradiance reaction is antagonistic to the low energy inductive reaction, whereas in seedling photomorphogenesis the two processes are synergistic.

The light reactions of phytochrome can be summarized in the following way:



where N is the photon fluence rate, and σ_1 and σ_2 are the photoconversion cross-sections. Light establishes a photoequilibrium between P_r and P_{fr} , expressed as the proportion of total phytochrome (P) in the form P_{fr} , ϕ . If a constant amount of phytochrome is assumed (there is very little evidence for P_r synthesis or P_{fr} destruction in seeds, although it is difficult to make direct measurements on phytochrome in the tissue of small seeds), then ϕ will be proportional to the amount of P_{fr} provided that the thermal reversion of P_{fr} to P_r is slow relative to the light reactions. However, this represents a dynamic condition of pigment interconversion, both reactions occurring simultaneously although with different rate constants. Thus for any given light condition there is a characteristic 'cycling rate' or 'photostationary flux'. The 'relative' cycling rate is here designated as H . Both ϕ and H can be calculated from the spectral photon distributions of the light source, using published¹² values of the phytochrome photoconversion cross-sections (σ_1 and σ_2) and the following equations:

$$\phi = \frac{\sum N_\lambda \sigma_{1\lambda}}{\sum N_\lambda \sigma_{1\lambda} + \sum N_\lambda \sigma_{2\lambda}}$$

$$H = (1 - \phi) \sum N_\lambda \sigma_{1\lambda} = \phi \sum N_\lambda \sigma_{2\lambda}$$

It is also possible to estimate ϕ and H for a particular light source by spectrophotometric measurements on dark grown seedling tissue, which is rich in phytochrome. Tissue is exposed to the light source for increasing periods of irradiation, and the

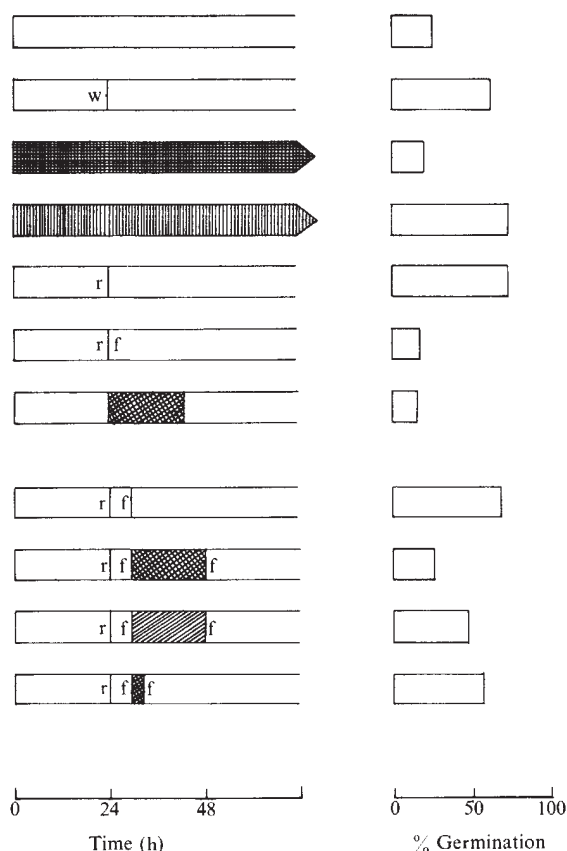


Fig. 1 Photocontrol of seed germination in *Sinapis arvensis*. Seeds irradiated and incubated at 15 °C. Treatments are indicated as follows: r = 5 min red light; f = 15 min far-red light; w = 15 min white incandescent light. □, Dark; ▨, high fluence rate white incandescent light; ▩, low fluence rate white incandescent light; ▤, high fluence rate light, $\phi = 0.27$; ▥, low fluence rate light, $\phi = 0.27$.

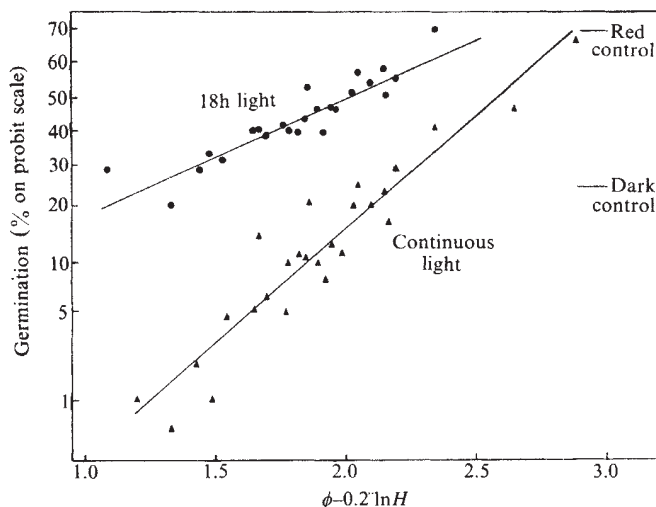


Fig. 2 Germination of *Sinapis* seeds in light of varying ϕ and H at 15 °C. Seven values of ϕ were used ranging from 0.05 to 0.70. Each point represents the mean percentage germination of four dishes of 50 seeds each. Standard errors were in the range 1 to 4, the average being 2. These were placed in a crossed gradient consisting of five 1.5 m 65/80W cool white (daylight) fluorescent tubes (with half their length blacked out) at one end (establishing a high ϕ value), and a 1-kW incandescent spotlight at the other end. The spotlight was placed behind a filter consisting of 10 cm of water, and one layer of no. 5A deep orange and one of no. 20 deep blue primary Cinemoid, which removed practically all irradiation below 700 nm and thus established a low ϕ value. Seeds were exposed either continuously from the start of imbibition until final germination was reached ($\blacktriangle$), or as part of the following regime: 24 h dark – 5 min red – 5 h dark – 15 min far-red – 18 h treatment – 15 min far-red – 3 d dark ($\bullet$). ϕ and H were determined by spectral measurement using a modified ISCO model SR spectroradiometer with a cosine-corrected remote probe. Spectrophotometric measurements of the P_{fr}/P ratio were made on dark-grown *Avena* coleoptiles which were kept on ice throughout exposure to the light. The coleoptiles were then packed into a specially designed cuvette under green safelight, and transferred to a Hitachi/Perkin-Elmer 156 digital dual wavelength spectrophotometer with a special actinic irradiation accessory.

proportion of phytochrome as P_{fr} measured in each case. ϕ is the P_{fr}/P ratio after a saturating irradiation. H can be calculated from ϕ and the time (t_1) to reach the half-saturation value of P_{fr}/P . For the reaction $P_r \rightarrow P_{fr}$:

$$k_1 = N\sigma_1 = \frac{\ln 2}{t_1} \phi$$

$$H = (1 - \phi)k_1 \\ = \frac{\ln 2(1 - \phi)\phi}{t_1}$$

In this study ϕ and H were varied by setting up a crossed gradient with two light sources at opposite ends of a constant temperature (15 ± 1 °C) growth room. There was a low ϕ value at one end of the room, and a high ϕ value at the other end. ϕ and H were determined at several positions along the gradient, both by spectral measurement and spectrophotometric measurement on coleoptile tips. The two independent methods of determining ϕ and H gave very similar values.

Thus ϕ and H can be used to describe the light environment in terms of the photoreactions of phytochrome. These values may not necessarily reflect the state of phytochrome within the seed, as this is affected by the transmission characteristics of the seed coat and internal tissues.

The photocontrol of *Sinapis* seed germination at 15 °C is summarized in Fig. 1. This shows the normal red/far-red reversible germination response, with a short far-red treatment inhibiting germination below the dark level. An 18-h period of high fluence rate light which establishes a ϕ value of 0.27

will reduce germination below this far-red level. If a 5-h dark period is given between red and far-red pulses, the seeds escape far-red reversion. This indicates that P_{fr} action, whatever this may entail, has been completed. However, it is still possible to block the product of P_{fr} action by giving an 18-h period of high fluence rate light after the escape from reversion, and low fluence rate light has a blocking effect as well, although reduced. The data show that the photoinhibition process is both time and fluence rate dependent.

There appears to be two processes controlling *Sinapis* seed germination. P_{fr} is essential for an early event in the processes leading to germination. However, a time- and fluence-rate-dependent process will block germination at a stage after the apparent completion of P_{fr} action. These two processes can thus operate sequentially, which can lead to the loss of photoinhibition so that the P_{fr} promoted process must be repeated. Thus in the natural light environment the two processes will operate simultaneously and antagonistically.

Seeds were sown in dishes along the crossed gradient to examine the relationship between germination and the two phytochrome parameters, ϕ and H . H was varied by shading the seed dishes with muslin or Cinemoid neutral density filters. The prolonged irradiation period was given both after the completion of P_{fr} action and continuously from the start of imbibition. It was found empirically that for a given ϕ value, the probit of germination was inversely proportional to the logarithm of H , and that the proportionality constant was ~ 0.2 for all ϕ values in both types of experiment. The straight line relationship between a mathematical expression combining ϕ and H and subsequent germination is illustrated in Fig. 2. This shows the antagonism between ϕ and H in the events leading to germination. From this relationship it is possible to predict the percentage germination of *Sinapis* seeds from a description of the light environment in terms of ϕ and H .

A theoretical 'action spectrum' can be generated from this relationship by choosing a particular level of response and calculating the value of H which will give this response for monochromatic irradiations across the spectrum. The absolute photon fluence rate required at each wavelength to achieve this response can be back-calculated from H . The reciprocals of these values when plotted against wavelength produce the action spectrum shown in Fig. 3. This should only deviate from an experimental action spectrum in those parts of the spectrum where the rate of P_{fr} dark reversion is appreciable compared with the rate of the photoreactions.

The striking point about this generated action spectrum is its similarity to experimental action spectra published for the high irradiance reaction in seeds^{7,9}. It is therefore reasonable

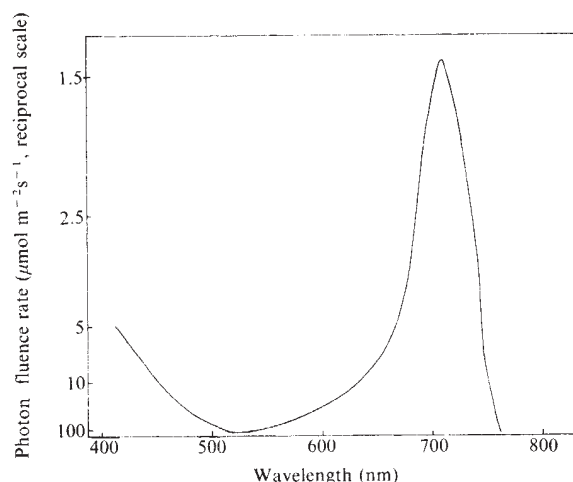


Fig. 3 Relationship between wavelength and calculated photon fluence rate giving half-maximum germination in *Sinapis* seeds. These were calculated using the expression $\phi - 0.2 \ln H = 1.93$, the latter being obtained from the data for 18-h irradiations given after escape from reversion.

to assume that these action spectra (strictly speaking these data for wavelength dependence are not true action spectra) arise because of the interaction of two aspects of phytochrome, the photoequilibrium ϕ and the photoconversion rate H . Further unpublished experiments using the seeds of *Plantago major* L. and *Bromus sterilis* L. support this view. Seeds of *Bromus sterilis* are apparently unique in that P_{fr} actually inhibits germination¹³, and experiments using prolonged irradiations with this species suggest that P_{fr} and H will interact such that both processes inhibit germination.

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Regeneration of normal and fertile plants that express octopine synthase, from tobacco crown galls after deletion of tumour-controlling functions

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Crown gall, a neoplastic transformation of plant cells, is caused by transfer and integration into nuclear plant DNA of T-DNA, a segment of the Ti plasmid of *Agrobacterium tumefaciens*^{1–8}. In plant cells, this T-DNA is expressed^{9–12} and determines the morphogenetic growth properties of crown gall cells^{12–16} and the synthesis of opines^{17–21}. The T-DNA in octopine crown galls is composed of either one or two fragments derived from a continuous Ti sequence. Thus far, all octopine tumour lines contain the TL fragment (14 kilobases (kb)), whereas TR-DNA (~6 kb) can be present as an additional sequence^{22–24}. Foreign DNA, inserted in the T region of a Ti plasmid, is co-transferred to the plant genome^{17,18,21}. However, an important requirement for the use of T-DNA as vectors for plant genetic engineering is the regeneration of normal plants which stably maintain, express and sexually transmit the transferred DNA. We report here the isolation, from tumour tissues induced by mutant Ti plasmids, of morphologically normal plants that produce octopine synthase in all tissues. This enzymatic activity is inherited as a dominant mendelian marker²⁵. These regenerated plants retained and expressed only the octopine synthase gene of the T-DNA.

On *Nicotiana tabacum*, *Agrobacterium* strains harbouring the mutant Ti plasmids, pGV2100 (ref. 15) and pGV2206 (ref. 21), induced small, greenish tumours covered with numerous shoots, whereas infection with the wild-type octopine plasmid resulted in large, white tumours without visible differentiation. Bacterium-free tumour tissue cultures induced by these mutant Ti

plasmids proliferate on hormone-free medium at the same rate as wild-type tumour cells and they synthesize octopine. The greenish aspect and the ability to form shoots were maintained after repeated subculturing.

In an initial experiment 250 shoots from 9 independent tumour lines induced by pGV2100 were tested, and one was found to synthesize octopine. This plant, rGV1 (ref. 25), was derived from tumour line IGV20 and developed normal roots on culture on hormone-free Murashige and Skoog²⁶ medium.

Out of 250 shoots, from 8 independent pGV2206 tumour lines incited on tobacco and growing at 25 °C, no octopine-positive shoots could be recovered. Teratomata formation by tumours induced by nopaline strains on tobacco was increased by incubation at 33 °C. Under these circumstances both normal shoots, able to form roots after separation from the tumour, and teratoma-like shoots unable to form roots were observed for the pGV2206-induced tumour lines. From these tumour lines 250 shoots were derived at 33 °C and analysed for the presence of octopine. One tumour line, IGV23, produced 4 octopine-positive plants in 54 shoots tested. Like rGV1, these regenerates, rGV2, rGV3, rGV4 and rGV5, were morphologically normal and could be transferred to soil. Physical analysis of the regenerates by Southern blotting hybridization revealed that they were identical (G. Gheysen, personal communication).

The octopine-positive regenerates obviously differ from suppressed nopaline teratoma shoots obtained from T37 nopaline tumour lines^{27–30} in the following aspects. (1) The shoots readily form a normal root system. (2) The shoots develop into plants which do not form spontaneous tumours and their cells are strictly dependent on exogenous hormones for division. (3) The regenerated plants respond to infection with agrobacterial strains carrying either wild-type or mutant Ti plasmids in an identical way to wild-type tobacco. (4) Earlier data, obtained with rGV1, showed that flower morphology and fertility were normal and that opine synthesis is completely retained through the meiotic process²⁵.

To determine whether all cells of the rGV4 express the octopine synthase gene, protoplasts were prepared from leaf mesophyll cells²⁵. Fifty independent calli were tested and found to be positive for octopine synthase activity³¹. To test whether the T-DNA inserts were at identical or different chromosomal loci in rGV1 and rGV4, these plants were crossed and the offspring was analysed. Using a semiquantitative LpDH assay²³ a number of offspring plants were identified presumably containing both T-DNA inserts. Two of these plants were castrated and fertilized with pollen from wild-type W38 tobacco. In one case 71% of the plantlets and in the other case 76% were found to be octopine-positive. These results indicate that the T-DNA inserts in rGV1 and rGV4 are genetically unlinked.

The T-DNA-derived transcripts in the regenerated plants and in the parental tumour tissues were compared with those present in the wild-type octopine tumour line A6-S1 (Fig. 1). The pGV2100-induced tumour line IGV20 clearly lacks transcript 2, and contains a very reduced level of transcript 1 (Fig. 1). The pGV2206-induced tumours lack both transcripts 1 and 2 and contain normal levels of the other TL transcripts¹⁶. In contrast, rGV1 only expressed transcript 3, which specifies octopine synthase²⁰ (Fig. 1). Four IGV23-derived regenerates, rGV2, rGV3, rGV4 and rGV5, were also found to express transcript 3 only, as ³²P-labelled cDNA, made towards their poly(A)⁺ RNA, hybridized solely with the T-DNA region overlapping with pTiB6S3 fragments *Sma*I-3b and *Eco*RI-7 (data not shown). Thus, the octopine synthase gene is the only T-DNA gene that remained expressed in the octopine-positive plants.

We analysed the T-DNA sequences in IGV20 (tumour line) and in rGV1 (regenerated plant) by DNA/DNA hybridizations. These revealed that the T-DNA in IGV20 is co-linear with the T-region of pGV2100. In rGV1 DNA, however, only probes from the right end of the TL-DNA hybridized and revealed a single 3.2-kb fragment in a *Hind*III digest (Fig. 2). This indicated that secondary rearrangements have deleted most, if not

Fig. 1 Analysis of T-DNA transcripts. Total RNA isolation and selection of poly(A)⁺ RNA fraction from leaves of rGV1, from callus of pGV2100-induced tumour line 1GV20 and from the A6-S1 suspension culture was according to ref. 10. Electrophoresis in 1.2% agarose after denaturation with glyoxal/dimethyl sulphoxide, transfer to diazobenzyloxymethyl paper, pretreatment of the filters and hybridization to ³²P-labelled T-DNA probes were performed as described elsewhere¹². Hybridization of poly(A)⁺ RNA from wild-type octopine tumour line A6-S1 against total T-region probe (a mixture of nick-translated pTiB6S3 *Bam*HI fragment 8 and *Hind*III fragment 1) shows the seven TL-encoded transcripts (numbered 1 to 7 according to diminishing size) (lane a). Hybridization of poly(A)⁺ RNA from 1GV20 against total T-region probe (lane b) and against *Bam*HI fragment 8, which contains the complete coding sequences for transcripts 5, 7, 2 and part of the coding sequences for transcript 1 (lane c), clearly indicate the absence of transcript 2, a very reduced level for transcript 1 and wild-type levels of transcripts 5, 7, 4, 6 and 3. Hybridization of poly(A)⁺ RNA from rGV1 against total T-region probe only lights up transcript 3 (lane d). The location of the seven TL-encoded transcripts on the physical map of the pTiAch5 T-region is from ref. 12. The direction of transcript 5 was determined from sequence data (J. Gielen, personal communication).

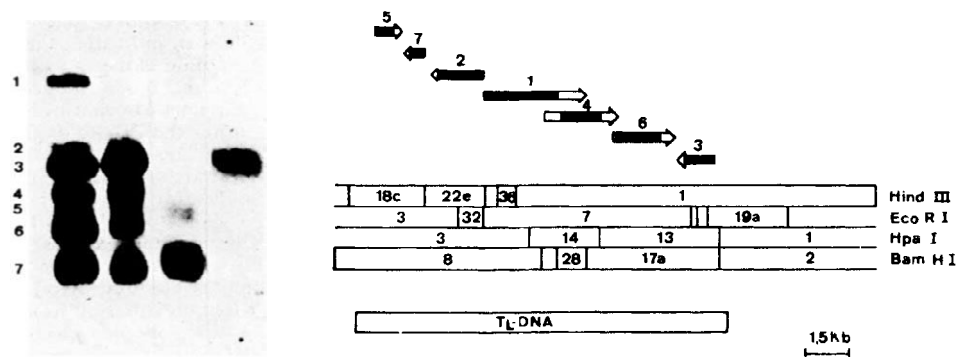
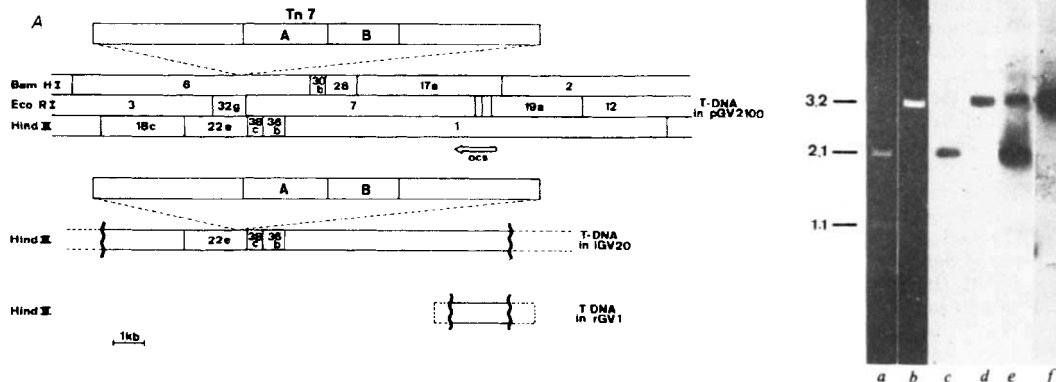


Fig. 2 Analysis of the T-DNA extent in 1GV20 and in rGV1. A, Genomic blotting. DNA was prepared from lyophilized 1GV20 tumour tissues and from rGV1 plants. Plant DNA (10 µg) was digested with *Hind*III and the fragments were separated by agarose gel electrophoresis, denatured and transferred to nitrocellulose filters. Filters were hybridized against ³²P-labelled clones containing the right part (pGV0201)³⁴ and the left part (pGV0153)³⁴ of the pTiAch5 T-region and against pGV26¹⁸, a ColE1 plasmid with a Tn7 insertion. The interpretation of the resulting hybridization patterns is shown, the T-region in pGV2100 is shown for comparison. A and B are internal *Hind*III fragments of Tn7 of, respectively, 2.7 and 2.25 kb¹⁵. The T-DNA in tumour line 1GV20 is co-linear with the T-region of the inducing pGV2100. The T-DNA borders have the same approximate location as that described for most of the octopine TL-DNAs studied thus far²²⁻²⁴. No hybridization was observed using probes pGV0153 and pGV26 against rGV1-DNA. This indicated that most, if not all, of the left part of the T-DNA and the Tn7 are absent in rGV1 DNA. Hybridization of *Hind*III-digested rGV1 DNA against pGV0201 revealed a single hybridization band of 3.2 kb. This band also lit up when the pTiAch5 *Bam*HI fragment 17 and *Hpa*I fragment 14, both internal T-DNA fragments situated at the right of the T-region, were used as probes. B, Genomic cloning. rGV1 DNA (50 µg) was digested to completion with *Hind*III and separated on a preparative low-gelling agarose gel. The DNA band corresponding to the 3.2-kb region was eluted from the gel³⁵. Charon phage 21A was grown as described by Blattner in the detailed protocol that accompanies the Charon λ phages. Phage DNA was extracted from the purified phage as described previously³⁶. The cohesive ends of the phage were annealed³⁶ and the phage DNA was digested with *Hind*III and treated with bacterial alkaline phosphatase to prevent *in vitro* packaging of the phage DNA without insert. 1 µg of insert DNA (±3.2 kb) was ligated with 10 µg Charon 21A phage DNA in 35 µl of ligase buffer³⁴. Five packaging reactions, each containing 2.2 µg of recombinant DNA, were performed with *in vitro* packaging extracts prepared as described elsewhere³⁷. *In vitro* packaged phages were preabsorbed to a fresh overnight BHB2600 bacterial culture³⁸ for 20 min at 37 °C³⁷, mixed with 0.7% top agar and plated at a density of 20,000 phage per 900-cm² plate. The plates were incubated at 37 °C for 14–16 h and refrigerated for at least 2 h before the nitrocellulose filters were applied. DNA was absorbed, denatured and bound to these filters as described previously⁴. Filters were prehybridized overnight at 42 °C in about 10 ml prehybridization mix (25 ml formamide, 2.5 ml 1 M HEPES pH 7.5, 2.5 ml 100×Denhardt³⁹, 7.5 ml 20×SSC⁴⁰, 1 ml 10 mg ml⁻¹ yeast RNA, water to 50 ml). Denatured *Escherichia coli* DNA (50–100 µg ml⁻¹) was included in the prehybridization mix. The prehybridization and subsequent hybridization were carried out in a sealed plastic bag. The filters were hybridized with agitation at 42 °C for 24 h in prehybridization solution containing ³²P-labelled pGV0201 as probe. After hybridization, the filters were washed two or three times, each time with shaking, for 1 h at 50 °C in 500 ml washing solution (0.1×SSC, 0.1% SDS), blotted dry and exposed to Fuji X-ray films with intensifying screens at –76 °C for 1–2 days. Plaques from the region of a plate corresponding to a positive signal on the autoradiogram were picked and resuspended in 0.5 ml λ phage buffer. The phage suspension was titrated and the plate containing ~100 plaques was rescreened. The process of picking positives and rescreening was repeated until ~90% of the plaques on a plate gave positive signals after screening. In this way, two independent, but identical clones were obtained. Phage DNA of these clones was prepared as described above and digested with *Hind*III. The 3.2-kb *Hind*III fragment was purified from low gelling agarose, digested with *Bam*HI and run in an agarose gel next to the *Hind*III fragment (lanes a, b). After transfer, the nitrocellulose filter was hybridized with purified *Bam*HI fragment 17a (lanes c, d) and subsequently with purified *Hind*III fragment 1 as probes (lanes e–g). The *Bam*HI fragment 17a only hybridized with the 2.1-kb fragment (lane c), whereas the *Hind*III fragment 1 hybridized with both the 2.1- and 1.1-kb fragments (lane f).



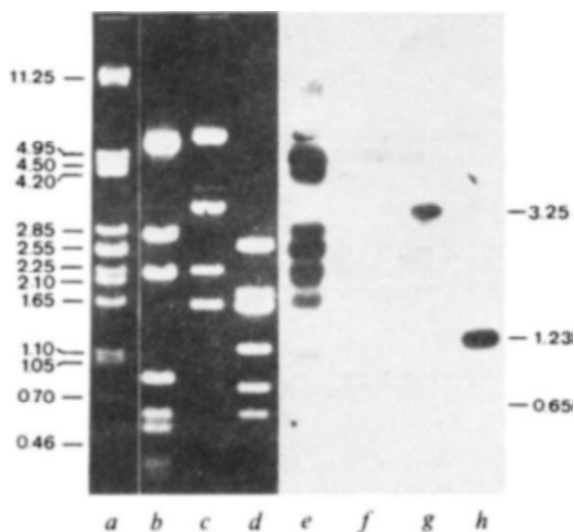


Fig. 3 T-DNA present in rGV1 mainly consists of sequences of the octopine synthase gene. Double digests, performed on different clones (lanes b–d) overlapping the whole TL-DNA, were blotted and hybridized with gcl rGV1-1 as a radioactive probe. No hybridization could be observed with the *Bam*HI–*Hind*III subfragments of *Bam*HI fragment 8 (lane f), and two out of three *Eco*RI–*Hpa*I subfragments of *Eco*RI fragment 7 (lane g). The two *Eco*RI–*Hpa*I subfragments which did not hybridize are the *Eco*RI–*Hpa*I fragment situated in *Bam*HI fragment 8 and the internal *Hpa*I fragment 14 of *Eco*RI fragment 7 (see Fig. 1). Hybridization of the genomic clone gcl rGV1-1 as probe against a *Bam*HI/*Sma*I double digest of *Bam*HI fragment 17a revealed that only the largest fragment hybridized (data not shown). To determine more precisely the end of the T-DNA in rGV1, a *Bam*HI/*Pvu*II double digest of *Bam*HI fragment 17a was hybridized with the genomic clone. Two of the four *Bam*HI–*Pvu*II subfragments lit up (lane g). This observation corresponds with the results obtained from sequence data which situated a *Pvu*II site in the 3'-untranslated region of the octopine synthase gene³². Lane a, λ digested with *Pst*I as molecular weight standard; lane b, *Bam*HI/*Hind*III double digest of clone pGV0153, containing the *Bam*HI fragment 8; lane c, *Eco*RI/*Hpa*I double digest of clone pGV0507³⁴, containing the *Eco*RI fragment 7; lane d, *Bam*HI/*Pvu*II double digest of clone pGV99³², containing the *Bam*HI fragment 17a; lanes e, f, g and h, hybridization patterns obtained from, respectively, lanes a, b, c and d, using the genomic clone gcl rGV1-1 as probe.

all, of the left and middle of the T-DNA. A single hybridization band of 3.2 kb also appeared on hybridization of *Hind*III-digested DNA from rGV1 offspring obtained after selfing a flowering rGV1 plant, indicating that the T-DNA sequences of rGV1 passed through meiosis without rearrangements.

To confirm further and document the conclusion that the T-DNA in the rGV plants had sustained extensive deletions, genomic clones were prepared from rGV1 DNA using Charon phage 21A as vector. Two independent but identical clones were obtained (gcl rGV1-1 and gcl rGV1-2). A *Hind*III/*Bam*HI double digest of the gcl rGV1-1 clone gave two fragments of 2.1 and 1.1 kb, hybridizing to a probe containing the *Hind*III fragment 1 (Fig. 2B). The observed *Hind*III fragment of 3.2 kb presumably contained the *Bam*HI site separating the T-region *Bam*HI fragments 17 and 2. Both the *Hind*III sites bordering this fragment must be derived from plant DNA sequences into which the T-DNA fragment is inserted. This conclusion was substantiated by demonstrating that (1) a probe containing *Bam*HI fragment 17a hybridized to the 2.1-kb but not to the 1.1-kb *Bam*HI–*Hind*III fragment (Fig. 2B); (2) clone gcl rGV1-1, when used as a probe against blotted TL-DNA fragments of pTiAch5, only hybridized with the right part of the TL-DNA (Fig. 3); (3) clone gcl rGV1-1, when used as a radioactive probe against a blotted *Eco*RI/*Bam*HI double digest of clone pGV105³² (containing the *Eco*RI fragment 19a of the TL-DNA region of pTiAch5), hybridized with both the

*Eco*RI–*Bam*HI fragments of the *Eco*RI fragment 19a (data not shown), indicating that the *Bam*HI site in the 3.2-kb *Hind*III genomic clone is indeed the site between *Bam*HI fragments 17a and 2; (4) the gcl rGV1-1 clone contains reiterated plant sequences bordering the remaining T-DNA sequences. Using either the 2.1-kb or the 1.1-kb *Hind*III–*Bam*HI subfragments as probes, in a Southern gel blotting hybridization against untransformed W38 tobacco DNA, a smear was obtained indicating that they both contained reiterated plant sequences.

This study proves that normal and fertile plants which still contain and express T-DNA sequences, can be regenerated from tobacco crown gall tumour cells. These 'transformed' plants are free of oncogenic properties and therefore fundamentally different from suppressed teratomata²⁷ or from opine-positive shoots lacking root formation and normal flower morphology³⁰. The latter shoots needed to be grafted and developed into abnormal plants containing most, if not all, of the T-DNA sequences. The rGV plants described in this study, however, were shown to have lost all tumour-controlling genes by a secondary deletion in their T-DNA. The inactivation of most of the *onc* genes could therefore be essential for the regeneration of normal plants in tobacco³³.

Tumours in which TL-DNA transcripts 2, or 2 and 1, are absent seem to provide favourable conditions for the formation of shoots from nontumorous cells and from rare cells carrying a mutated T-DNA. Furthermore, our results do not distinguish whether the mutated T-DNA results from a secondary deletion after T-DNA integration, or from a mutant T-DNA present in only a few bacteria and transferred to a fraction of the transformed plant cells. If the latter were the case, we expect that Ti plasmids carrying a mutated T-DNA, similar to the one in rGV1, will efficiently transfer and integrate this T-DNA without inducing tumorous growth. The use of these mutated Ti plasmids as suitable vectors for gene transfer into dicotyledonous plants will then require: (1) appropriate conditions to regenerate plants from undifferentiated callus cultures; (2) a selective method to recover 'transformed' cells; indeed, it is likely that T-DNA-positive cells will be present only as a minority amongst wild-type cells.

It is important to stress that genes, such as opine synthases, introduced into plants via modified Ti plasmids, are stably maintained not only in somatic cells, but also through meiosis. Furthermore, independent insertions were shown to take place at different loci.

Finally, the finding that T-DNA-containing plants of the rGV1 type are fully susceptible to Ti plasmid-mediated transformation opens up the possibility of successive insertions of different genes in the same plant.

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Exon shuffling: mapping polymorphic determinants on hybrid mouse transplantation antigens

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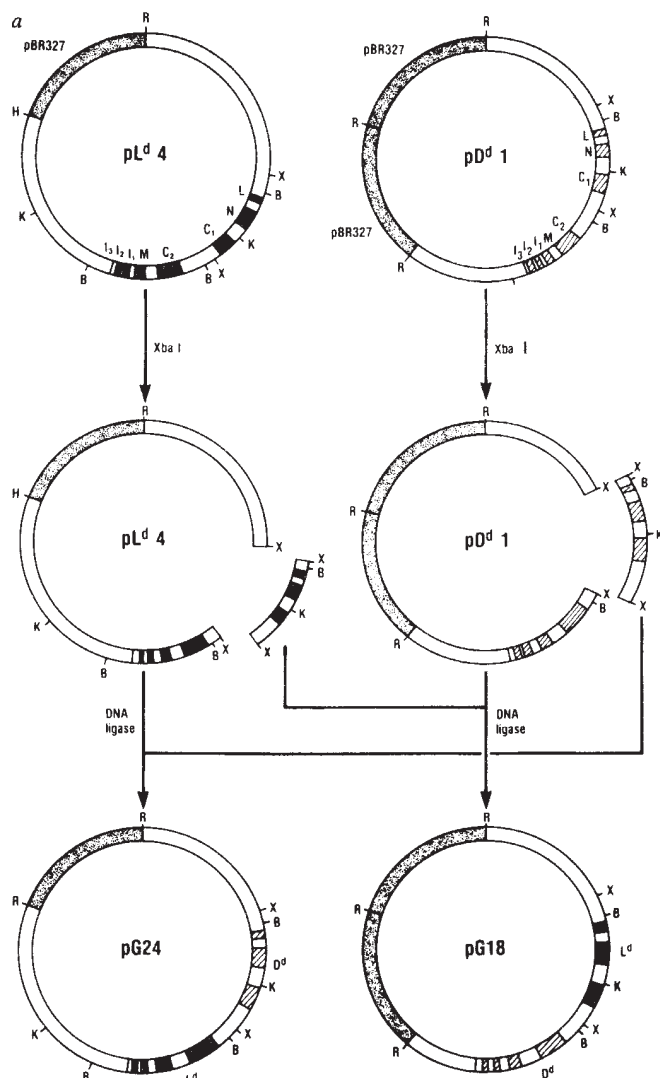
The mouse major transplantation antigens H-2K, H-2D and H-2L are highly polymorphic cell-surface glycoproteins which may serve as recognition elements in cell-cell interactions¹. Each antigen possesses a number of alloantigenic determinants defined by antisera of various specificities. Recently, monoclonal antibodies have been produced which redefine and extend our knowledge of these determinants^{2,3}, but structural information has not yet been correlated with the serological definition of the antigens. We have previously reported the molecular cloning of genes for H-2L^d and H-2D^d transplantation antigens from the BALB/c mouse and the expression of these genes in mouse L cells^{4,5}. To localize the serological determinants to discrete regions of the H-2 protein, we have now constructed new H-2 antigen genes by joining together fragments of the H-2L^d and H-2D^d genes. In L cells, these genes direct the synthesis of hybrid H-2 proteins and by using monoclonal antibodies of defined specificities, we have mapped classically defined serological specificities to structurally defined domains of the transplantation antigen protein. We conclude that polymorphic determinants recognized by monoclonal antibodies are located in functionally distinct portions of the protein.

We obtained genomic clones containing H-2L^d and H-2D^d genes from a BALB/c mouse genomic library^{4,5}. The DNA

sequence of the entire 4.5-kilobase (kb) H-2L^d gene has been determined (refs 4, 6 and G.A.E., unpublished data). The three exons at the 5' end of the H-2D^d gene have been sequenced and the derived amino acid sequence agrees precisely with the known H-2D^d protein sequence⁵. These genes were introduced into thymidine kinase(tk)-deficient mouse L cells by DNA-mediated gene transfer^{7,8} and two cloned L cell lines were obtained which expressed H-2L^d and H-2D^d determinants in the presence of the parental H-2^k background. Thus, the H-2 phenotypes of these cell lines are H-2K^k, D^k, L^d (cell line T4.8.3) and H-2K^k, D^k, L^d (cell line T1.1.1). Using a panel of 13 monoclonal antibodies having H-2 specificities, we screened these cell lines for reactivity (Table 1). All three H-2L^d-specific monoclonal antibodies reacted with T1.1.1 cells but not with T4.8.3 cells. However, those antibodies reacting with antigens encoded by the D region of the H-2^d haplotype could not distinguish between determinants shared between H-2L^d and H-2D^d and those unique to H-2D^d, because of a lack of appropriate recombinant strains⁹. The availability of transformed cell lines separately expressing H-2D^d and H-2L^d antigens has allowed us to assess the precise specificities of the antibodies. Six monoclonal antibodies were found to be specific for the H-2D^d-producing cell line. The remaining three antibodies showed significant reactivity with both H-2L^d and H-2D^d, indicating specificity for a shared determinant (Table 1).

In order to more precisely define the structural basis of the allodeterminants recognized by these antibodies as well as allorecognition by T lymphocytes, we constructed hybrid H-2 genes by combining exons from the cloned H-2L^d and H-2D^d genes. Several restriction endonuclease cleavage sites, including two *Xba*I sites, were conserved between the two sequences. After subcloning the respective genes into plasmid vectors, *Xba*I fragments containing the 5' coding region of the genes were excised, exchanged and re-ligated with the 3' end of the genes using T₄ DNA ligase (Fig. 1). This reconstruction resulted in two hybrid genes. The gene carried on the pG18 plasmid contains the three 5' exons (L, N and C1) of H-2L^d and the 3' exons of the H-2D^d gene. pG24 contains the first three exons of H-2D^d and the 3' end of H-2L^d (Fig. 1b). If correctly expressed, the products of these genes would contain the N and C1 external domains of one transplantation antigen molecule and the C2 domain of the other.

To determine whether these hybrid genes could be expressed in mouse fibroblasts, pG18 and pG24 DNA was introduced into L(tk⁻) cells by DNA-mediated gene transfer^{4,5}. Cloned cell lines from the resultant transformants were screened with a panel of monoclonal antibodies specific for H-2D^d and H-2L^d determinants. IgG class monoclonal antibodies were tested for binding by radioimmunoassay (Table 1a). One of the anti-H-2L^d antibodies, 28.14.8, reacted specifically with pG24-containing cells (T37.2.1) but not with pG18-containing cells, indicating that the determinant recognized by this antibody depends on the presence of the C2 domain of the H-2L^d molecule for expression. Although conformational interactions of widely separated portions of the molecule could be responsible for unmasking the monoclonal antibody determinant, the most likely explanation is that the 28.14.8 antibody recognizes a determinant physically located on the C2 domain of the molecule. Similarly, the determinants recognized by antibodies 34.5.8, 28.8.6 and 34.1.2 map to the N or C1 domains of the H-2D^d protein, the specificity of antibody 34.2.12 maps to the C2 domain of H-2D^d, and the specificity of antibody 30.5.7 maps to the N or C1 domains of H-2L^d. IgM class monoclonal antibodies, screened by flow microfluorimetry (Table 1b) show that H-2D^d specificities 34.4.21 and 23.5.21 map to the N or C1 regions of H-2D^d and the 23.10.1 determinant maps to the N or C1 domains of the H-2L^d molecule (Table 1b). Monoclonal antibodies 34.4.20, 27.11.13 and 28.11.5, which show cross-reacting specificities, react in all cases with both hybrid gene products. However, significant quantitative differences in binding suggest that these determinants are not identical. For example, antibody 34.4.20 binds significantly better to H-2D^d



and the pG24 gene product (N and C1 of H-2D^d; C2 of H-2L^d) than to H-2L^d or the pG18 gene product. Monoclonal antibodies specific for N-C1 or C2 domains have been used to immunoprecipitate molecules from both T37.1.3 and T37.2.1 (G.A.E., unpublished). These immunoprecipitated proteins have molecular weights of 45,000 daltons and associate with β_2 -microglobulin, consistent with the predicted hybrid protein structures.

This study demonstrates the feasibility of *in vitro* genetic recombination to generate hybrid, and thus allogeneically novel transplantation antigens which are expressed in a normal fashion on the cell surface. Thirteen determinants defined by monoclonal antibodies were active in the hybrid gene products. No determinants present in the two parental gene products were lost in either of the two hybrid gene products. Therefore the domain structure of the transplantation antigens may also define a functional separation of polymorphic antigenic sites. The location of antigenic determinants can be correlated with the domain structure of the molecule. Polymorphic determinants recognized by monoclonal antibodies are located on the N, C1 and C2 domains. Although the C2 domain shows significantly less variability in amino acid sequence than the N and C1 regions^{10,11}, polymorphic alloantigenic determinants may be located in this region of the protein also. Interestingly, monoclonal antibody 28.14.8 was originally found to be cross-reactive with the H-2D^b antigen. This study maps this determinant to the C2 domain and recent amino acid sequence data demonstrate that the C2 domains of H-2L^d and H-2D^b are identical¹¹. In addition, monoclonal antibodies 28.14.8 and

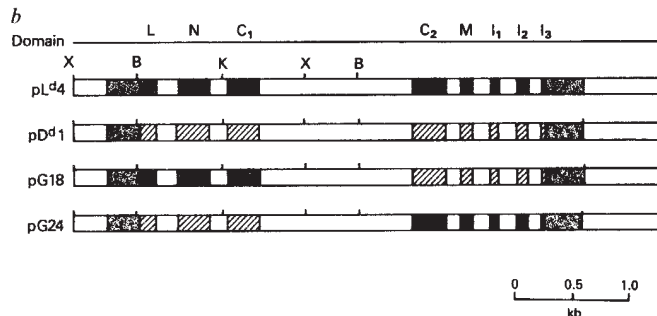


Fig. 1 Construction of recombinant genes from the cloned H-2D^d and H-2L^d genes. *a*, Fragments of the bacteriophage DNA containing H-2 genes were subcloned into plasmid vectors for gene manipulation experiments. A 12-kb *EcoRI*-*HindIII* fragment from Ch4AH-2L^d (ref. 4) was subcloned into the *EcoRI* and *HindIII* sites of pBR327 (ref. 19), producing the plasmid pL^d4. Similarly, a subclone containing the H-2D^d gene, pD^d1, was prepared by subcloning the 8-kb *EcoRI* fragment of Ch4AH-2D^d (ref. 5) into the *EcoRI* site of pBR327. (As an artefact of this cloning procedure, pD^d1 contains two copies of pBR327 joined at the *EcoRI* sites in the same orientation.) Recombinants between the H-2L^d and H-2D^d genes were constructed by cleaving both plasmids with *Xba*I. The resultant fragments were purified by electrophoresis on 1% low melting point agarose gels and elution at 65 °C, followed by purification on DE-23 columns. The 1.6-kb *Xba*I fragment from pL^d4 was inserted into the 15-kb *Xba*I fragment of pD^d1 using 1 unit of T₄ DNA ligase in 10 mM Tris-HCl pH 7.6, 1 mM dATP, 0.5 mM EDTA, 6 mM MgCl₂ and 50 mM NaCl for 6 h at 14 °C. The 15-kb fragment was treated with bacterial alkaline phosphatase before ligation to prevent recircularization. The ligated DNA was reintroduced into *E. coli* LE392 by CaCl₂-induced transfection¹⁷. Colonies were screened for the insert having the correct orientation by 'mini-DNA' preparations¹⁸ and the resulting hybrid plasmid, pG18, extensively mapped by restriction endonuclease cleavage to confirm the reconstruction. Similarly, pG24 was constructed by ligating the 1.6-kb *Xba*I fragment from pD^d1 to the 15-kb fragment from pL^d4. *b*, The resulting hybrid genes were recombined at the *Xba*I site located within the 1.3-kb intervening sequence separating the exons coding for the C1 and C2 domains. The hybrid H-2 gene carried on pG18 contains the first three exons, corresponding to the L, N and C1 domains, of H-2L^d and the following five exons of H-2D^d. The gene on pG24 contains the L, N and C1 coding regions of H-2D^d and the 3' domains of H-2L^d. Cross-hatched areas represent the regions coding for H-2L^d; solid regions represent those coding for H-2D^d domains. Stippled areas represent pBR327 (*a*) or the 5' and 3' untranslated regions of the H-2 mRNA (*b*). L, N, C1, C2, M, I1, I2 and I3 refer to the regions of the protein encoded by each exon labelled by analogy to the H-2K^b prototype structure¹⁰. X, *Xba*I; B, *Bam*HI; H, *Hind*III; K, *Kpn*I; R, *Eco*RI cleavage sites.

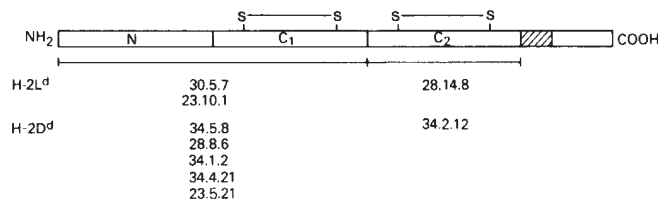


Fig. 2 Localization of polymorphic determinants on the external domains of H-2 proteins. This diagram summarizes the reactivity of H-2L^d- and H-2D^d-specific monoclonal antibodies with hybrid H-2 genes. The reacting monoclonal antibody is listed in relation to the globular subunit of the protein to which it binds. The determinants recognized by monoclonal antibodies 30.5.7 and 28.14.8 have previously been shown to correlate with the classical serological specificities H-2.65 and H-2.64, respectively^{2,12,13}.

30.5.7 correlate with serological specificities H-2.64 and H-2.65, respectively, originally defined by classical serology^{2,12,13}, which map to structurally distinct portions of the H-2L^d molecule (Fig. 2). Thus, the spatial relationships of polymorphic

Table 1 Monoclonal antibody binding to hybrid H-2 gene products

a	Antibody reactivity*									
	Monoclonal antibody Specificity	16.1.2 <i>K^kD^k</i>	34.4.20 <i>D^dL^d</i>	27.11.13 <i>D^dL^d</i>	30.5.7 <i>L^d</i>	28.14.8 <i>L^d</i>	34.5.8 <i>D^d</i>	28.8.6 <i>D^d</i>	34.1.2 <i>D^d</i>	34.2.12 <i>D^d</i>
Cell (gene)										
DAP-3		3,049	120	119	110	115	125	118	124	150
T1.1.1 (<i>L^d</i>)		2,024	1,816	1,212	2,925	2,335	107	95	157	116
T4.8.3 (<i>D^d</i>)		2,534	4,748	2,119	88	140	4,292	3,820	5,022	3,713
T37.1.3 (<i>L^dD^d</i>)		2,536	1,850	1,703	4,219	161	169	184	211	3,799
T37.2.1 (<i>D^dL^d</i>)		2,016	4,262	3,016	113	4,193	4,319	3,706	3,933	171
Antibody reactivity*										
b	Monoclonal antibody Specificity	34.4.21 <i>D^d</i>	23.5.21 <i>D^d</i>	23.10.1 <i>L^d</i>	28.11.5 <i>D^dL^d</i>					
Cell (gene)										
DAP-3		28.0	25.8	24.5	28.1					
T1.1.1 (<i>L^d</i>)		36.5	23.4	284.2	202.5					
T4.8.3 (<i>D^d</i>)		297.0	242.8	26.5	335.3					
T37.1.3 (<i>L^dD^d</i>)		31.4	23.5	238.1	73.2					
T37.2.1 (<i>D^dL^d</i>)		856.2	338.3	19.2	342.5					

Cloned H-2 genes were introduced into thymidine kinase-deficient mouse L cells (DAP-3) by DNA-mediated gene transfer using the herpes virus thymidine kinase gene¹⁶ as a selectable marker. The cloned cell lines T1.1.1, containing an H-2L^d gene⁴ and T4.8.3, containing an H-2D^d gene⁵, have been described elsewhere. These cloned cell lines have been maintained in continuous culture for over 6 months without loss or alteration of H-2 expression. Monoclonal antibodies specific for H-2D^d and/or H-2L^d determinants have been characterized previously^{2,9}. a, IgG class monoclonal antibodies having specificities for H-2L^d or H-2D^d were screened for binding to transformed L cells containing H-2L^d H-2D^d or one of the two hybrid genes pG18 or pG24. Screening was performed by radioimmunoassay in Falcon flat-bottom 96-well microtitre plates. 10⁶ cells were added to each well and incubated overnight at 37 °C in Dulbecco's modified Eagle's medium supplemented with glutamine, hypoxanthine, aminopterin, thymidine and 10% fetal calf serum⁴. After washing, 50 µl of a 1:1 dilution of hybridoma supernatant were added and the cells were incubated for 60 min at 4 °C. The cells were extensively washed, ¹²⁵I-labelled goat anti-mouse IgG added to each well, and the cells incubated for an additional 60 min at 4 °C. After extensive washing, cells were lysed with 1% Triton X-100, supernatants collected with a Titertek supernatant collection system, and radioactivity determined in a γ-spectrometer. Results are expressed as c.p.m. ¹²⁵I bound above background. Each value represents the mean of triplicate samples with a standard deviation of less than 3% of the mean. Monoclonal antibody 28.8.6 was previously reported to react only with H-2K^b and H-2D^b (ref. 3). Additional cross-reactivities with H-2D^d have been found by radioimmunoassay and flow microfluorimetry (K.O., unpublished). b, IgM class monoclonal antibody binding was determined by flow microfluorimetry as described^{4,5} using fluorescein-conjugated goat F(ab')₂ anti-mouse F(ab')₂. The results represent mean fluorescence normalized to gain 1. The fluorescent profile showed a single population of stained cells. Standard deviation of the mean fluorescence was less than 60 fluorescence units.

* Only the reactivity relevant to this study is shown.

determinants previously assessed only by competitive binding of monoclonal antibodies² may, using these techniques, be correlated with distinct molecular structures.

Monoclonal antibodies against H-2 specificities have been shown to specifically block cell-mediated lympholysis by T cells activated against H-2 allodeterminants^{14,15}. Specifically, monoclonal antibody 28.14.8 has been shown to block both allogeneic and H-2-restricted cell-mediated lympholysis¹⁴. As shown here, the determinant recognized by antibody 28.14.8 is located on the C2 domain, a region which may, therefore, sterically affect H-2 antigen recognition by T lymphocytes. The use of H-2 antigens modified by genetic engineering coupled with *in vitro* mutagenesis makes feasible the complete structural and functional definition of allodeterminants on transplantation antigens and their recognition by T cells.

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Amplification of immunoglobulin λ constant genes in populations of wild mice

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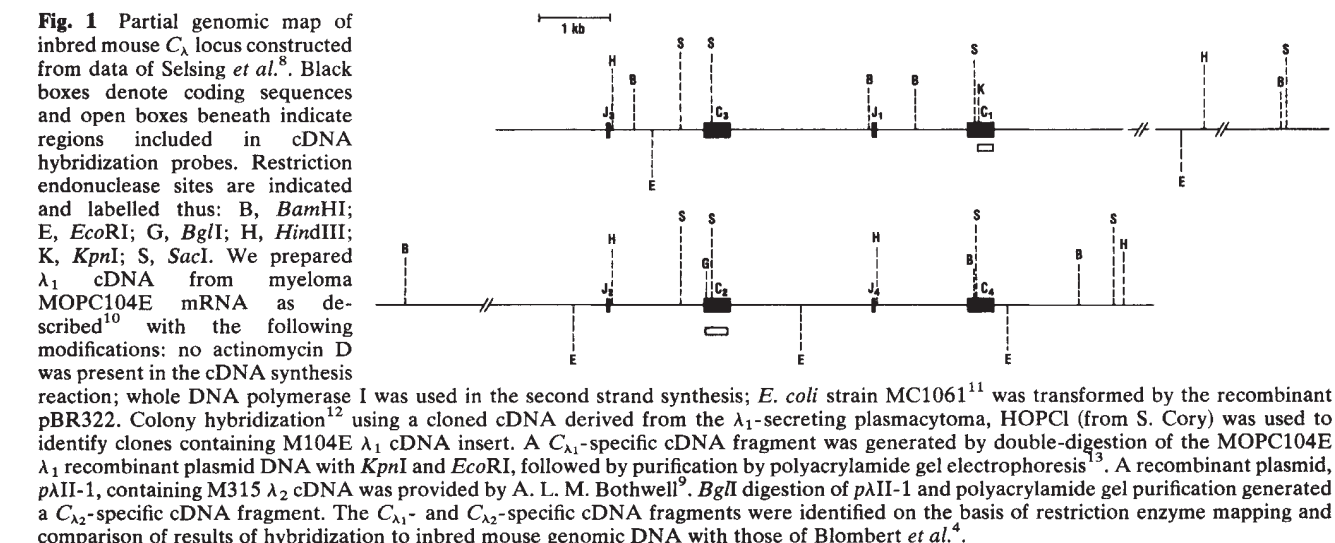
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The λ immunoglobulin light chain (Igλ) locus of BALB/c inbred mice consists of two variable region gene segments (*V_λ*)^{1–3}, and four constant region gene segments (*C_λ*)^{1,2,4,5}. Each *C_λ* gene segment is associated with a unique joining segment (*J_λ*)^{2,4–7}, and they are organized in two paired units, *J₃C₃–J₁C₁* and *J₂C₂–J₄C₄* (refs 4, 8). Using cDNA probes specific for *C_λ* and *C_λ* (ref. 9) we have analysed the genomic organization of the *C_λ* gene segments in wild-derived and inbred strains of mice. Although Southern blots of the genomic DNA of inbred mice show a constant pattern of hybridization, wild-derived mice show a high degree of variation in the number, size and intensity of hybridizing fragments. We have now found that, per haploid genome, mice of a *Mus musculus musculus* stock isolated from Sladecovce, Czechoslovakia (CzII) have at least 12 *C_λ* segments, and mice of a *Mus musculus domesticus* stock 'Centre-ville Lights' from Centreville, Maryland (CL) have at least 8 *C_λ* segments. There appears to have been relatively recent amplifications of the *C_λ* gene segments in wild mice.

The immunoglobulin *C_λ* *J₃C₃–J₁C₁* and *J₂C₂–J₄C₄* paired units share a similar internal organization^{4,5,8} (Fig. 1). Considerable sequence homology exists between *C_λ* and *C_λ*; somewhat



less homology is shared by C_{λ_1} and C_{λ_4} (ref. 8). Hybridization of C_{λ_1} cDNA to *Eco*RI digested inbred mouse liver genomic DNA identifies a 10 kilobase (kb) fragment containing the C_{λ_1} gene segment, and a fainter 2.8 kb fragment containing the C_{λ_4} gene segment (Fig. 2a, lane 1). There is no hybridization to C_{λ_2} or C_{λ_3} (ref. 4). Similarly, C_{λ_2} cDNA hybridizes to 10-kb and 3.2-kb fragments containing the C_{λ_3} and C_{λ_2} gene segments, respectively, but not to C_{λ_1} or C_{λ_4} (Fig. 2b). These *Eco*RI band patterns are representative of those observed for all inbred strains examined thus far: AKR/N, BALB/cAn π , BALB/cJ, C57BL/Ka, C58, C3H/HeN, SJL/J, SJA, SWR/J, DBA/2, PL/J, BSVS, RF/J, 129/J, ST/bJ, SM/J, RIII, AuSSJ, CBA/ICR and CBA/T6T6. In striking contrast to the patterns observed with inbred strains are those obtained in many wild mouse populations, represented in Fig. 2 by two examples, CL (lane 2) and CzII (lane 3). The CL mouse *Eco*RI patterns contain six fragments that strongly hybridize to the C_{λ_1} probe and six that hybridize to the C_{λ_2} probe, while the same probes hybridize to eight and seven fragments, respectively, of CzII DNA digested with *Eco*RI.

Several explanations might account for the extra bands seen in CL and CzII DNA. Incomplete digestion by the endonuclease could give rise to more fragments hybridized to the cDNA probes. The same patterns for both inbred and wild-derived mice, however, have been seen repeatedly in stringently controlled digestion, blotting and hybridization conditions. Moreover, incomplete digestion of the DNA surrounding the C_{λ_3} - C_{λ_1} unit should give rise to fragments larger than the 10 kb fragments seen in inbred mice. Instead, all but one of the

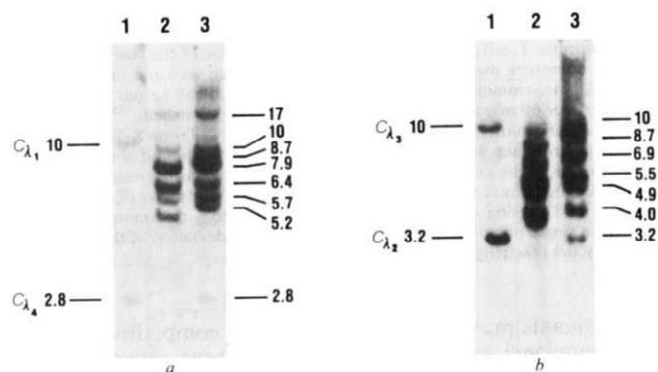


Fig. 2 *Eco*RI digested BALB/c, CL and CzII genomic DNA hybridized to C_{λ_1} - and C_{λ_2} -specific cDNA probes. Genomic DNA was prepared from mouse liver by homogenization in 5% citric acid, isolation of nuclei, lysis at 65 °C in 50 mM Tris-HCl pH 9.5, 100 mM EDTA, 1% SDS, sequential treatment with 500 μ g ml⁻¹ pronase and 10 μ g ml⁻¹ RNase, phenol extraction and ethanol precipitation. The data shown were generated by digestion of 10 μ g of each DNA sample with restriction endonuclease (3–5 U per μ g DNA) for 2–12 h at 37 °C, electrophoresis on 0.7% agarose followed by blotting onto nitrocellulose¹⁴. Before hybridization, blots were soaked in 6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M Na citrate), 10 $\times$ Denhardt's solution¹⁵, 0.1% SDS and 60 μ g ml⁻¹ sheared salmon sperm DNA for 2–4 h at 65 °C. 1 $\times$ 10⁷ c.p.m. of C_{λ_1} or C_{λ_2} cDNA labelled by nick-translation¹⁶ with [α -³²P]dCTP to $\sim$ 2 $\times$ 10⁸ c.p.m. per μ g DNA was hybridized to the genomic DNA blot overnight at 65 °C, in the same conditions as for prehybridization. The hybridized blots were washed once in 10 $\times$ Denhardt's solution, 2 $\times$ SSC, 0.1% SDS, 1 mM EDTA for 30 min at 65 °C, twice in 2 $\times$ SSC, 0.1% SDS, 1 mM EDTA for 30 min at 65 °C and twice in 0.2 $\times$ SSC, 0.1% SDS, 1 mM EDTA for 30 min at 65 °C. *a*, BALB/c (1), CL (2) and CzII (3) liver DNA hybridized to C_{λ_1} cDNA. *b*, Hybridization of the same DNAs to C_{λ_2} cDNA. The sizes (kb) of the BALB/c and CzII bands are indicated. Sizes were determined by comparison of the mobility of the hybridized fragment to a standard curve generated from linear regression analysis of 35 gels where *Hind*III fragments of phage λ DNA were used as DNA size markers. BALB/c fragments containing C_{λ_1} , C_{λ_2} , C_{λ_3} and C_{λ_4} genes were identified by comparison to published photographs⁴ and restriction enzyme maps⁸. To ascertain that the hybridizations were indeed to C_λ coding regions and not to sequences related to the 3' untranslated region, the λ_1 cDNA probe was fractionated to generate a fragment that contained coding region only and no 3' untranslated sequence. When this fragment was labelled and hybridized to *Eco*RI digested BALB/c, CL and CzII genomic DNA under conditions as described above, we observed the same number of C_λ bands as seen in *a*, thus confirming hybridization to coding sequence.

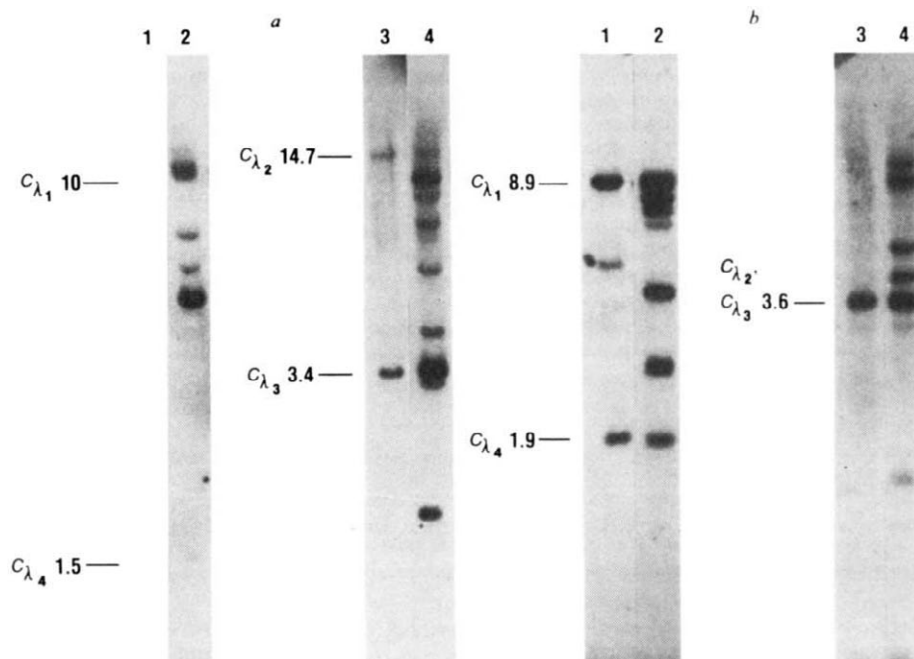
Table 1 Number of fragments strongly hybridizing to C_{λ_1} and C_{λ_2} probes

Genomic DNA source	cDNA probe	<i>Eco</i> RI	<i>Bam</i> HI	<i>Hind</i> III	<i>Sac</i> I
BALB/c (Inbred)	C_{λ_1}	2	1*	2	3
	C_{λ_2}	2	2	2	1
<i>M. musculus domesticus</i> (Centreville)	C_{λ_1}	6	4	5	6
	C_{λ_2}	7	5	5	5
<i>M. musculus musculus</i> (Sladecovce)	C_{λ_1}	8	6	6	8
	C_{λ_2}	7	9	6	6

Number of DNA fragments that strongly hybridize to C_{λ_1} and C_{λ_2} probes. The number of strongly hybridizing fragments generated under conditions as described previously is indicated. Highly intense bands, which may indicate the presence of multiple copies of a gene fragment, are counted as one band.

* Only one of the two *Bam*HI fragments hybridizes strongly. The C_{λ_4} gene fragment hybridizes very weakly and was therefore not counted.

Fig. 3 Restriction endonuclease digestion (a, *Bam*HI and b, *Sac*I) of BALB/c (lanes 1, 3) and CzII (lanes 2, 4) DNA hybridized to C_{λ_1} cDNA (lanes 1, 2) and C_{λ_2} cDNA (lanes 3, 4). Digestion, hybridization and wash conditions were as described in Fig. 2 legend. Sizes of inbred mouse DNA fragments are indicated.



additional bands are smaller than 10 kb. Similarly, incomplete digestion products of the C_{λ_2} – C_{λ_4} unit would be larger than the 2.8 kb fragment of inbred mouse DNA hybridizing to the C_{λ_1} cDNA probe. Although the additional bands in CL and CzII are larger than 2.8 kb, the intensity of those bands is so much greater than that of the inbred mouse 2.8 kb fragment that it seems unlikely they are the result of incomplete digestion of that unit. As further evidence of completeness of digestion, when the same filters are stripped of labelled C_{λ} probe and rehybridized to probes independent of C_{λ} (for example, V_{λ} , C.L.S., manuscript in preparation), multiple bands are not present in CL and CzII DNA. Therefore, the patterns seen with the C_{λ} probes may be explained by the occurrence of multiple C_{λ} sequences in the DNA of CL and CzII.

Mutations generating *Eco*RI sites in the coding region of any of the inbred mouse C_{λ} gene segments would also give rise to additional fragments. However, the size range and number of the CL and CzII fragments is inconsistent with such an interpretation. In the unlikely event that multiple *Eco*RI sites have arisen in the coding region, which is only 318 base pairs (bp) in length, the fragments resulting from *Eco*RI digestion would, with the possible exception of those immediately adjacent to flanking regions, be very small and difficult to detect by Southern hybridization. A third basis for multiple bands in wild mice might be polymorphism and heterozygosity. Polymorphism does appear to exist between CL and other mice derived from the same population in Centreville, Maryland (C.L.S. and M.P., unpublished data). The difference between individuals, however, is not seen as multiple bands in one case and few in the other, but involves a difference of only one or two bands, depending on the restriction enzyme used. Heterozygosity may exist, because these colonies are maintained in an outcrossed fashion. However, all CzII individuals so far examined have the same pattern as do (BALB/c $\times$ CzII)_{F1} animals, which show BALB/c bands plus all CzII bands. Further, pooled genomic DNAs of mice derived from various localities in Eastern Europe and South-East Asia show a complex hybridization pattern similar to that of CzII. Even if polymorphism exists, it does not explain the large increase in number of C_{λ} bands seen in some of the wild populations. Instead, the patterns of C_{λ} fragments strongly suggest that more C_{λ} genes are present in these mice than in inbred strains.

To substantiate the multiple gene copy interpretation, we digested the same DNAs with *Bam*HI, *Hind*III, *Kpn*I and *Sac*I and hybridized with C_{λ_1} and C_{λ_2} probes. Multiple intense bands

were visible in the wild mouse DNAs for all enzymes used, while inbred mice consistently had few bands (Table 1). Figure 3 shows examples of hybridizations to inbred (lanes 1, 3) and CzII (lanes 2, 4) DNA digested with *Bam*HI (Fig. 3a) and *Sac*I (Fig. 3b). The hybridization intensity of some of the wild mouse DNA fragments is substantially greater than that of the fragment containing the inbred mouse gene homologous to the cDNA probe. For example, the 7.9 kb *Eco*RI band of CzII is much darker than the 10 kb band of BALB/c, which contains the C_{λ_1} gene (Fig. 2a). Similar highly intense bands are visible in *Bam*HI digested CzII DNA hybridized to either the C_{λ_1} or the C_{λ_2} cDNA probe. The inbred mouse *Bam*HI fragments containing the C_{λ_1} and C_{λ_2} genes do not hybridize as strongly. This suggests that more than one copy of C_{λ} gene sequence is present in the strongest hybridizing fragments. On the basis of the intensity and number of additional fragments generated by digestion with five different restriction endonucleases, we conclude that CL and CzII mice have more C_{λ} genes than do inbred mice. In light of the fact that the inbred mouse C_{λ_1} and C_{λ_4} sequences do not cross-hybridize with the C_{λ_2} and C_{λ_3} sequences, it is apparent that the CL and CzII mice have multiplied sets of both C_{λ_1} -analogous and C_{λ_2} -analogous genes. Exact determination of the number of C_{λ} genes in the wild mice is not yet possible, but the independent C_{λ_1} and C_{λ_2} cDNA hybridization results indicate the presence of at least 8 in CL mice and 12 in CzII mice.

The four C_{λ} genes of inbred mice are presumed to be the product of duplication of a C_{λ_2} – C_{λ_4} unit^{4,6,8}, with C_{λ_2} as ancestor of C_{λ_2} and C_{λ_3} , and C_{λ_4} of C_{λ_1} and C_{λ_4} . Possible mechanisms of amplification include duplication of single C_{λ_x} or C_{λ_y} genes, sequential duplication of a C_{λ_x} – C_{λ_y} unit, or simultaneous duplication of multiples of the unit. When both the number and intensity of C_{λ} bands in CL and CzII are considered, the increase in number of C_{λ_x} and C_{λ_y} genes appears concordant, which more strongly supports a unit mechanism of duplication than the single gene duplication mechanism. Although it is not yet clear whether analogues to both inbred mouse C_{λ} units are involved, it seems that an analogue to the C_{λ_2} – C_{λ_1} unit has participated in the duplication process, as indicated by the intensity of bands hybridizing to C_{λ_1} cDNA. In duplication of a unit containing a C_{λ_x} gene that strongly hybridizes to the C_{λ_1} cDNA probe, there would be concordant duplication of a C_{λ_x} gene that strongly hybridizes to the C_{λ_2} cDNA probe. The hybridization patterns of CL and CzII are consistent with such a prediction.

The additional C_λ genes described in CL and CzII may be ancestral genes that segregated away from the progenitors of inbred strains, or they may represent a recent amplification of C_λ in some wild mouse populations. If the CL and CzII mice carry ancestral conserved genes, evolutionary drift of the DNA sequence would probably generate weaker hybridization bands than are observed. Thus, the strength of hybridization of inbred mouse $C_{\lambda 1}$ and $C_{\lambda 2}$ cDNA probes to CL and CzII C_λ genes seems to support recent amplification. There is evidence that duplication of C_λ genes has already occurred fairly recently in the mice from which inbred strains are derived, as indicated by the homology of the two C_λ units^{4,6,8}.

It is generally accepted that a single C_κ gene is adequate for expression of a large repertoire of immunoglobulin κ light

chains in mice, so the role of multiple C_λ genes is not obvious. If J_λ amplification occurs concurrent with C_λ amplification, immunoglobulin λ diversity might ultimately be influenced. It may be that amplification of C_λ genes is associated with amplification of V_λ genes as well, but as the location of mouse V_λ genes relative to C_λ genes remains unknown, this possibility cannot yet be assessed.

We are currently preparing genomic clones from CzII mice to elucidate further the nature of the C_λ amplification at the DNA level. In addition, experiments at the RNA and protein levels are in progress to ascertain whether the additional C_λ genes are expressed.

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Inherited deletion of immunoglobulin heavy chain constant region genes in normal human individuals

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The existence of specific probes for human genes makes it feasible to study genetic abnormalities, both inherited and acquired, at the level of the genome. In this respect, the antibody genes of man are of particular interest as they represent a multigene family expressed in many leukaemias and immunodeficiency diseases. Furthermore, selective deficiency of immunoglobulins has been described in healthy individuals¹. Normally, human adults express five types of immunoglobulin—IgM, IgD, IgG, IgE and IgA (defined by the class of heavy chain constant region). Subclasses are also known in IgG (IgG1, IgG2, IgG3 and IgG4) and IgA (IgA1 and IgA2) in which the immunoglobulins contain $\gamma 1$, $\gamma 2$, $\gamma 3$ or $\gamma 4$ and $\alpha 1$ or $\alpha 2$ C_H regions, respectively. Recently, a healthy Tunisian person was described who showed abnormal patterns of immunoglobulin expression². The serum immunoglobulin of this individual, designated TAK3, was confined to IgM, IgD, IgG3, IgE and IgA2. We have now used cloned C_H -gene probes to study the DNA of TAK3 as well as two brothers, also Tunisian but apparently unrelated to the individual TAK3, and who show a similar immunoglobulin abnormality. We found that in these cases there seems to have been a large chromosomal deletion which includes three γ genes, an α gene and a pseudo- ϵ gene. This deletion accounts for the simultaneous absence of certain H-chain subclasses. These results illustrate that the human immunoglobulin gene locus is capable of undergoing rapid change, which is particularly apparent within small populations in which consanguinity is common.

The patterns of hybridization with C_H -gene probes of the DNA from TAK3, and the brothers TOU and EZZ were studied by Southern filter hybridization, and compared with DNA from individuals with normal immunoglobulin expression (both of Tunisian and European origins) plus a person, TAK14 (the daughter of TAK3), who is heterozygous for the immunoglobulin abnormality. The state of the γ genes in TAK3 and

TOU was examined using a $\gamma 3$ probe (clone p3.6RH4.2, which contains a 3.6-kilobase (kb) *EcoRI*–*HindIII* fragment encompassing the whole $\gamma 3$ gene coding region³) and a $\gamma 4$ probe (clone pBRH4.1, which contains an analogous fragment encompassing the $\gamma 4$ gene³). These probes cross-hybridize with the genes for the various γ subclasses. A high degree of restriction fragment polymorphism has been observed in the γ genes; both the $\gamma 4$ probe (Fig. 1a) and the $\gamma 3$ probe (Fig. 1b) detect between five and seven hybridizing *Bam*HI fragments as exemplified by genomic DNAs from different individuals including a placental DNA obtained in Cambridge (Fig. 1b, lane 4) and two other Tunisian DNAs, designated LAT and BOU (Fig. 1a, lanes 1, 4). Common 9-, 11- and 12-kb fragments were present in all these DNAs, and in one DNA an additional 23-kb band was detected (Fig. 1a, lane 4) which we also observed in other Tunisian DNAs (data not shown).

When we analysed hybridization of the $\gamma 4$ probe with *Bam*HI-digested DNA from TAK3, we observed only two bands (8 and 10.5 kb; Fig. 1a, lane 3) whereas hybridization with the $\gamma 3$ probe showed only the 10.5-kb fragment (Fig. 1b, lane 2). Presumably, this 10.5-kb *Bam*HI fragment detected in TAK3 DNA represents the $\gamma 3$ gene (because only the IgG3 subclass is expressed in TAK3). The unusual size of this fragment may result from an altered restriction site which occurred during the deletion event. The origin of the second, more weakly hybridizing component could be the pseudo- γ gene^{3,4} or even a partially deleted γ gene. Neither of the bands observed in TAK3 corresponds with the fragments detected in any of the other DNAs except TAK14 DNA (Fig. 1a, lane 2; b, lane 3) which is heterozygous for the immunoglobulin defect found in TAK3. The heterozygosity of TAK14 is demonstrated by the presence of a normal pattern of five bands plus the two unusual bands detected in TAK3 DNA. These data suggest that the simultaneous absence of $\gamma 1$, $\gamma 2$ and $\gamma 4$ subclasses in the homozygous individual TAK3 is due to an extensive deletion of the region of chromosome 14 which carries the γ genes^{5–7}. The hybridization of the $\gamma 3$ probe to *Bam*HI-digested TOU DNA is also shown in Fig. 1b (lane 1). Interestingly, a similar γ deletion has occurred in TOU to that which occurred in TAK3, except that we could only detect the 10.5-kb hybridizing band in TOU DNA. These results with γ -gene probes show that two individuals displaying abnormal patterns of γ -globulin expression seem to do so because of inherited deletions of the respective γ genes.

A further investigation of the C_H -gene pattern in the TAK, TOU and EZZ DNAs was made using μ - and δ -specific probes (Fig. 2) plus ϵ - and α -specific probes (Fig. 3), and the latter two gave further evidence for gene deletions. The probes used

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in Fig. 2 were C75p1.2 (a 1.2-kb *Eco*RI fragment of a human clone, carrying the C_μ coding regions, cloned into pACYC184⁸) and M13RP2 (a 1.5-kb *Eco*RI-*Pst*I fragment of a human clone containing part of the δ gene cloned into M13mp8). All five DNA samples analysed with the μ probe possessed 17-kb hybridizing *Bam*HI fragments (Fig. 2a) but in addition TAK14 and TAK3 showed a hybridizing band of 8 kb. The occurrence of this latter band in TAK3 (who is thought to be homozygous for the C_H locus²) is surprising; the most obvious conclusion must be that a duplication of μ has occurred in TAK DNA. No such putative duplication has occurred in the homozygous individuals EZZ or TOU, where we find only the 17-kb *Bam*HI restriction fragment (Fig. 2a, lane 5). A completely normal pattern of hybridization between TAK and TOU DNAs was found when we used the δ probe (Fig. 2b), as each of these DNAs display a 11-kb hybridizing *Bam*HI fragment found in 9 out of 10 control DNAs analysed (latter data not shown).

The ϵ and α genes in the various human DNAs were analysed using as hybridization probe for ϵ , the ϵ 1.2BP25 clone (a 2.1-kb *Bam*HI-*Pst*I fragment, derived from the clone ϵ 1.2, containing the entire human ϵ coding region⁹) and as an α probe the clone α 2XP8 (a 600-base *Xho*I-*Pst*I fragment of an α -gene cloned in M13mp8¹⁰). The restriction pattern most frequently seen for ϵ -gene hybridization in human DNA (Fig. 3) is the presence of three *Bam*HI fragments (2.7, 6 and 9 kb)^{9,11,12}. This pattern is exemplified in Fig. 3a, lane 3, by the DNA sample LAT. A frequently observed polymorphism is the presence of a fourth, 7.5-kb band⁹, which is present in DNA sample BOU (Fig. 3a, lane 5). It has recently been demonstrated that the 2.7-kb fragment carries the active ϵ gene^{9,11,12} whereas the 6- and 9-kb fragments represent pseudogenes called $\psi\epsilon$ 1 and $\psi\epsilon$ 2, respectively^{10,11}. When we examined the patterns of hybridization of the ϵ probe to both TAK and TOU DNAs we again

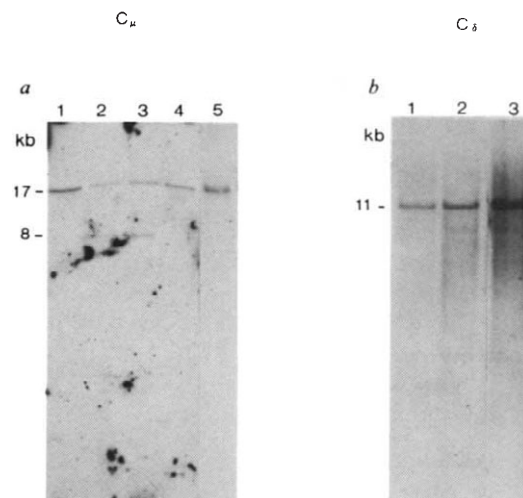


Fig. 2 Southern hybridization of C_μ and C_δ probes to genomic DNA from various sources. The DNA digests were: a, probe: μ ; slot 1, LAT; slot 2, TAK14; slot 3, TAK3; slot 4, placenta; slot 5, EZZ. b, Probe: δ ; slot 1, TAK3; slot 2, TAK14; slot 3, TOU. **Methods:** Genomic DNA was digested to completion with *Bam*HI, fractionated and blotted from 0.8% agarose gels onto cellulose nitrate and hybridized (as in Fig. 1) to nick-translated C75p1.2 (a) and M13RP2 (b) probes. Phage λ DNA cut with *Hind*III was used as molecular weight markers.

found evidence for gene deletion (Fig. 3a, lanes 1, 4). Both TAK3 and TOU DNAs show the presence of both the active ϵ gene and $\psi\epsilon$ 2 but no $\psi\epsilon$ 1. This observation is consistent with the deletion of the latter from these genomes because this gene is present in other DNAs (see, for example, LAT and BOU, lanes 3 and 5) including TAK14 who is heterozygous for the abnormality (Fig. 3a, lane 2).

The α -gene hybridization pattern observed using the α 2XP8 clone with TAK and TOU is shown in Fig. 3b. In this experiment we used a sample designated TAK92 (a non-blood relative of TAK3) as the control DNA: hybridization of the α probe with TAK92 (Fig. 3b, lane 3) shows two hybridization components (2 and 1.2 kb) in common with European DNAs tested simultaneously (latter data not shown). Partial nucleotide sequencing data¹⁰ indicate that the 2- and 1.2-kb fragments contain α 2 and α 1 coding sequences, respectively. The α -gene hybridization to DNA from TOU and TAK3 (Fig. 3b, lanes 1, 2) showed, in both cases, that the smaller of the hybridization components was absent, consistent with the presence of the α 2 gene and deletion of the α 1 gene from the genome of TAK3 and TOU individuals.

The Southern hybridization results presented here show that Tunisian individuals who exhibit simultaneous absence of IgG1, IgG2, IgG4 and IgA1 subclasses carry deletions for three γ genes (presumably γ 1, γ 2 and γ 4), a pseudo- ϵ gene ($\psi\epsilon$ 1) and an α gene presumed to be α 1. These gene deletions account for the lack of specific immunoglobulin expression in these individuals. The precise location of the gene deletion in these people need not necessarily be identical but it does seem likely that switch or S-sequences are the key to the deletion event. The S-sequences are groups of short tandemly repeated units which are probably involved in the mechanism of the class switch and have been implicated in the formation of a mouse myeloma deletion mutant¹³. A likely hypothesis to explain the deletions described here is that illegitimate recombination has occurred within the germ line between non-adjacent S-sequences with resulting deletion of the intervening C_H genes. Presumably, such deletions occasionally occur in all human populations but would not normally be detected because they do not seem to be harmful (the person designated TAK3, for instance, is a healthy 75-yr-old woman). However, in populations where frequent consanguinity occurs the probability of

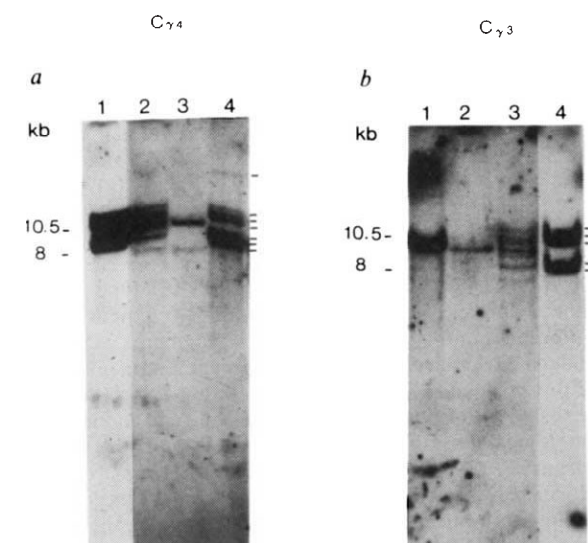


Fig. 3 Southern hybridization of C_γ 3 and C_γ 4 probes to DNAs from individuals showing serum immunoglobulin abnormalities. a, Probe: γ 4; slot 1, LAT; slot 2, TAK14; slot 3, TAK3; slot 4, BOU. b, Probe: γ 3; slot 1, TOU; slot 2, TAK3; slot 3, TAK14; slot 4, placental DNA.

Methods: DNA was digested to completion with *Bam*HI, the digests were fractionated on 0.8% agarose gel, and the DNA was transferred to cellulose nitrate filters¹⁴. LAT and BOU individuals were described previously¹⁵. The hybridization was carried out for 2 days at 65°C using 6×SSC, 0.1% SDS, 0.2% Ficoll 400, 0.2% bovine serum albumin (BSA), 0.2% polyvinylpyrrolidone (PVP) and 50 μ g ml⁻¹ sonicated, denatured, salmon sperm DNA^{16,17}, followed by washes in 1×SSC, 0.1% SDS at 65°C before autoradiography at -70°C with prefogged X-ray film¹⁸. Probes were nick-translated to a specific activity of $\sim 10^8$ c.p.m. per μ g. Size estimations (shown on the left of each panel) were made using λ cut with *Hind*III and the lines on the right of each panel signify location of restriction fragments discussed in the text.

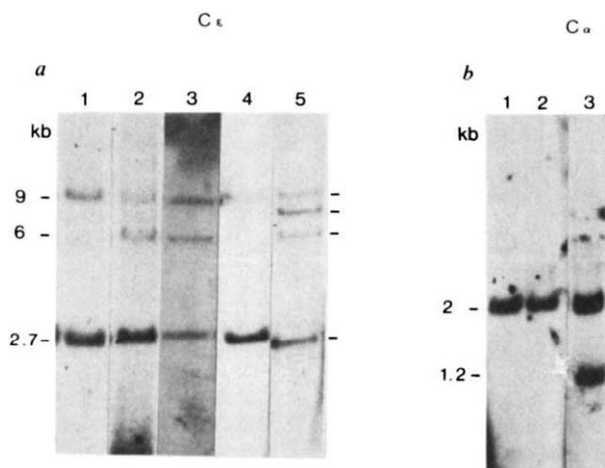


Fig. 3 Southern blot hybridization of C_ϵ and C_α probes to genomic DNA digested with *Bam*HI (a) or *Pst*I (b). The DNA digests were: a, Probe: ϵ ; slot 1, TAK3; slot 2, TAK14; slot 3, LAT; slot 4, TOU; slot 5, BOU. b, Probe: α ; slot 1, TOU; slot 2, TAK3; slot 3, TAK92.

Methods: Complete digests were separated on 0.8% agarose (a) or 1.4% agarose (b), blotted and hybridized (as in Fig. 1) with C_ϵ probe (a) or C_α probe (b). The size of fragments (given on the left of each panel) were estimated relative to λ phage DNA cut with *Hind*III. The lines on the right of a mark the positions of fragments discussed in the text.

homozygosity increases and such individuals are readily detected during random immunoglobulin haplotype screening.

Finally, the consistent deletion pattern of C_H genes in TAK, TOU and EZZ allows a link between the absence of C_H genes and the overall ordering of the human C_H genes described in the accompanying paper¹⁰. Two groups of cosmid clones have been described which seem to encompass $\gamma 3$ – $\gamma 1$ – $\psi \epsilon 1$ – $\alpha 1$ (region A) and $\gamma 2$ – $\gamma 4$ – ϵ – $\alpha 2$ (region B)¹⁰. As it seems that the deletions described here include $\gamma 1$, $\gamma 2$, $\gamma 4$, $\psi \epsilon 1$ and $\alpha 1$ genes, the most probable order for the groups of cosmid clones is 5' region A–region B 3' as the deletions presumably start downstream of $\gamma 3$ (region A) and end upstream of the active ϵ gene (region B). We are now using gene cloning studies to clarify these issues as well as the nature and localization of the points of deletion within the C_H genes.

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Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change

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Several different transforming genes have been observed in the DNA of a variety of tumours and tumour cell lines of human and rodent origin by the ability of these genes to induce morphological transformation in NIH 3T3 cells^{1–5}. The transforming gene found in a human bladder carcinoma cell line, T24, is *H-ras-1*, the human homologue of the Harvey sarcoma virus oncogene (*v-H-ras*)^{6–9}. In the present study we have compared the *H-ras-1* genes cloned from T24 and normal human DNA. The *H-ras-1* gene cloned from T24 DNA induces transformation in NIH 3T3 cells, while the same gene cloned from normal cellular DNA does not. The functionally significant difference between the transforming and normal genes appears to be a single base mutation, which produces an amino acid change in the sequence of the proteins that the genes encode.

We had previously isolated transforming sequences from NIH 3T3 cells that had been transformed with DNA from T24 bladder carcinoma cells¹⁰. These sequences had undergone some rearrangement during gene transfer into NIH 3T3 cells. As a result of passage in NIH 3T3 cells, the transforming gene might have undergone less readily detectable secondary changes altering its biological activity. Therefore, in order to properly compare the transforming sequences of T24 to their normal counterparts, we directly cloned the *H-ras-1* sequences from λ libraries constructed from T24 DNA and human placental DNA. From the T24 library we obtained seven independent isolates, all of which contained a 6.2-kilobase (kb) *Bam*HI *H-ras-1* fragment with an identical restriction endonuclease pattern. From the placental library, we obtained clones of two types: in three (P3 type), *H-ras-1* sequences were carried on a 6.7-kb *Bam*HI fragment; in the four others (P1 type), *H-ras-1* sequences were carried on a 8.3-kb *Bam*HI fragment (see Fig. 1). Differences in the size of the *Bam*HI fragments were expected due to restriction endonuclease fragment length polymorphism about this gene in the human population¹⁰.

We tested all of our isolates for the ability to induce foci of morphologically transformed NIH 3T3 cells on DNA mediated gene transfer. DNA from all T24 isolates of *H-ras-1* efficiently transformed NIH 3T3 cells, while DNA from all placental isolates failed to induce foci. This difference must be the result of differences in DNA sequence. To facilitate our search for these differences, we tested the transforming activity of chimaeric genes. The restriction endonuclease *Xba*I cleaves the *Bam*HI fragment containing the *H-ras-1* gene into one small and one large fragment (see Fig. 1). The large and small fragments were purified from each of the three cloned genes (types T24, P1 and P3), mixed and ligated in various combinations and finally cleaved with *Bam*HI. All (and only) molecules containing the small T24 *Bam*HI/*Xba*I fragment were capable of inducing transformed foci in NIH 3T3 cells (see Table 1). To further limit the area of our search we used restriction endonuclease *Mst*II, which cleaves the small *Bam*HI/*Xba*I fragment once. The 1.7-kb *Bam*HI/*Mst*II fragment and the 0.24-kb *Mst*II/*Xba*I fragment from the T24 or P3 *H-ras-1* genes, and the large *Xba*I/*Bam*HI fragment from the P3 gene were purified, mixed and ligated in various combinations, and then cleaved with *Bam*HI. All molecules containing the 0.24-kb *Mst*II/*Xba*I fragment from T24 induced transformed foci in

NIH 3T3 (see Table 1). The sequence changes in this region are therefore sufficient to confer the transforming capacity on the P3 H-*ras*-1 gene. A few foci are seen in one other combination of fragments (see Table 1) but we hesitate to ascribe significance to this.

We sequenced the 245-bp *Mst*II/*Xba*I fragment in its entirety from P1, P3 and T24 H-*ras*-1 genes. The sequences were identical at all but two nucleotide positions (see Fig. 1). These lie within the first coding exon of the H-*ras*-1 gene as determined by a comparison of genomic sequences to those of an H-*ras*-1 cDNA clone. (O.F. *et al.*, manuscript in preparation).

We found that P1 and P3 H-*ras*-1 genes encoded glycine (GGC) at amino acid 12, while the T24 gene encodes valine (GTC) at that position, the result of a single G → T nucleotide change. We also noted that while all three genes encode histidine at amino acid 27, the codon usage differs (see Fig. 1). Because this latter change does not alter the protein sequence we believe it is not functionally significant (see below).

The above data suggest that an amino acid substitution in the T24 H-*ras*-1 gene product generates a protein with greatly enhanced transforming activity. To test this prediction, we have introduced the T24 and placental H-*ras*-1 genes into NIH 3T3 cells using the pSV2gpt vector of Mulligan and Berg¹¹. In this way, we obtained 10 xgprt⁺ lines containing the T24 H-*ras*-1 gene and 16 lines containing the P3 H-*ras*-1 gene, as determined by Southern filter blot hybridization (see Fig. 2). All ten of the lines containing the T24 H-*ras*-1 gene appeared morphologically transformed, while only two of the lines containing the P3 H-*ras*-1 gene had any of the features we associate with morphological transformation. To measure more precisely the degree of growth transformation we examined the ability of several lines to grow in agar suspension (see Fig. 2). To correlate degree of transformation with gene expression, we measured H-*ras*-1 RNA by filter hybridization and H-*ras*-1 protein by immunoprecipitation of ³⁵S-methionine labelled cell lysates (see Fig. 2). The human H-*ras*-1 gene encodes a 21,000-MW protein (p21) which cross-reacts with antibody to the Harvey *v-ras* p21 (given by M. Furth and E. Scolnick).

In these studies, the only difference we can discern between the expression of the P3 and T24 H-*ras*-1 genes is the potency of their respective protein products. Highly transformed cell lines containing the T24 H-*ras*-1 gene (XT1 and XT3) contain

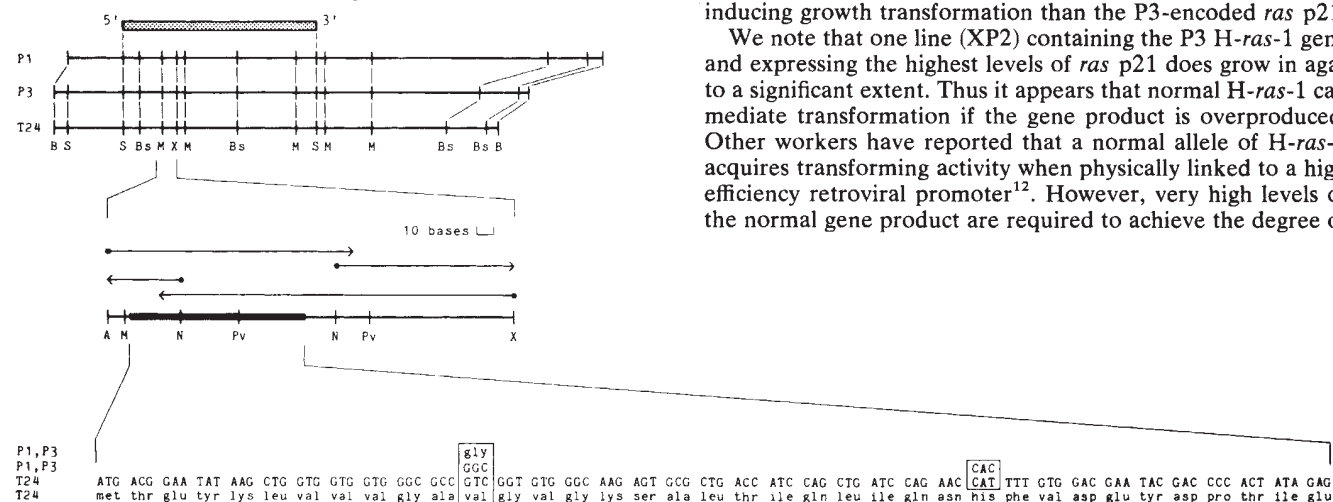


Fig. 1 Structural differences between human H-*ras*-1 genes. The *Bam*HI fragment containing the H-*ras*-1 gene was cloned from placental DNA and T24 DNA libraries constructed in the L47.1 cloning vector¹³. Phage libraries were constructed by the method of Hohn and Murray¹⁴ and screened without prior amplification by the method of Benton and Davis¹⁵. Restriction maps of the three gene types (P1 and P3 from placenta, and T24 from T24 cells) are shown above. In the region of the 2.9-kb *Sst*I fragment (shaded area) which contains a complete H-*ras*-1 gene (unpublished data) the restriction maps are similar. The 245-bp *Mst*II/*Xba*I fragment, which contains the functionally significant structural differences, was sequenced by the method of Maxam and Gilbert¹⁶ in entirety for all three genes. The sequencing strategy is shown in the middle panel. Restriction fragments were labelled at their 3' ends with the large fragment of DNA polymerase I¹⁷. All regions were sequenced at least once on both strands. The bold line indicates the first coding exon of 37 amino acids, determined by sequencing a T24 H-*ras*-1 cDNA clone (O.F. *et al.*, manuscript in preparation). In the bottom panel is shown the DNA sequence of T24 in this coding region and the predicted amino acid sequence. Where P1 and P3 differed from T24 are enclosed in boxes. Restriction endonucleases used were *Ava*I (A), *Bam*HI (B), *Bst*EII (Bs), *Mst*II (M), *Nar*I (N), *Pvu*II (Pv), *Sst*I (S) and *Xba*I (X).

Table 1 Focus induction with chimaeric H-*ras*-1 genes

(5') <i>Bam</i> HI/ <i>Xba</i> I	<i>Xba</i> I/ <i>Bam</i> HI(3')	Foci per 0.1 µg*
T24	T24	358
T24	P1	142
T24	P3	80
P1	T24	0
P1	P1	0
P3	T24	0
P3	P3	0

(5') <i>Bam</i> HI/ <i>Mst</i> II†	<i>Mst</i> II/ <i>Xba</i> I†	<i>Xba</i> I/ <i>Bam</i> HI(3')†	Foci per 0.02 µg‡
T24	T24	P3	88
T24	P3	P3	2
P3	T24	P3	163
P3	P3	P3	0

The T24, P1 and P3 *Bam*HI fragment containing the H-*ras*-1 gene was cleaved with *Xba*I and the respective 5' (*Bam*HI/*Xba*I) and 3' (*Xba*I/*Bam*HI) halves of the genes (see Fig. 1) purified from agarose gels as previously described²⁵. The various 5' and 3' halves were mixed in equimolar ratios in the combinations indicated above, ligated with T4 DNA ligase at a DNA concentration of 100 µg ml⁻¹, and then digested with *Bam*HI. The amount of material in the correct molecular form was then estimated by ethidium bromide staining after electrophoresis in agarose gels.

* Approximately 0.1 µg of the chimaeric genes was mixed with 90 µg of NIH 3T3 carrier DNA and added to 3 plates of NIH 3T3 as a calcium phosphate precipitate as previously described⁴. Morphologically altered foci of NIH 3T3 cells were scored after 14 days.

† The 1.7-kb *Bam*HI/*Mst*II and the 0.24-kb *Mst*II/*Xba*I fragments were purified from the T24 and P3 *Bam*HI fragments (see Fig. 1), mixed in equimolar ratio with the 4.8-kb P3 *Xba*I/*Bam*HI fragment, ligated with T4 DNA ligase at a DNA concentration of 100 µg ml⁻¹ and then cleaved with *Bam*HI. The amount of material in the correct molecular form was then estimated by ethidium bromide staining after gel electrophoresis.

‡ Approximately 20 ng of the chimaeric genes were mixed with 180 µg of NIH 3T3 carrier DNA and added to six plates of NIH 3T3. Foci were scored after 14 days.

far less *ras* p21 than do minimally transformed lines expressing the normal *ras* gene (XP1, XP3). We estimate from this that the T24-encoded *ras* p21 is at least 100 times more potent at inducing growth transformation than the P3-encoded *ras* p21.

We note that one line (XP2) containing the P3 H-*ras*-1 gene and expressing the highest levels of *ras* p21 does grow in agar to a significant extent. Thus it appears that normal H-*ras*-1 can mediate transformation if the gene product is overproduced. Other workers have reported that a normal allele of H-*ras*-1 acquires transforming activity when physically linked to a high efficiency retroviral promoter¹². However, very high levels of the normal gene product are required to achieve the degree of

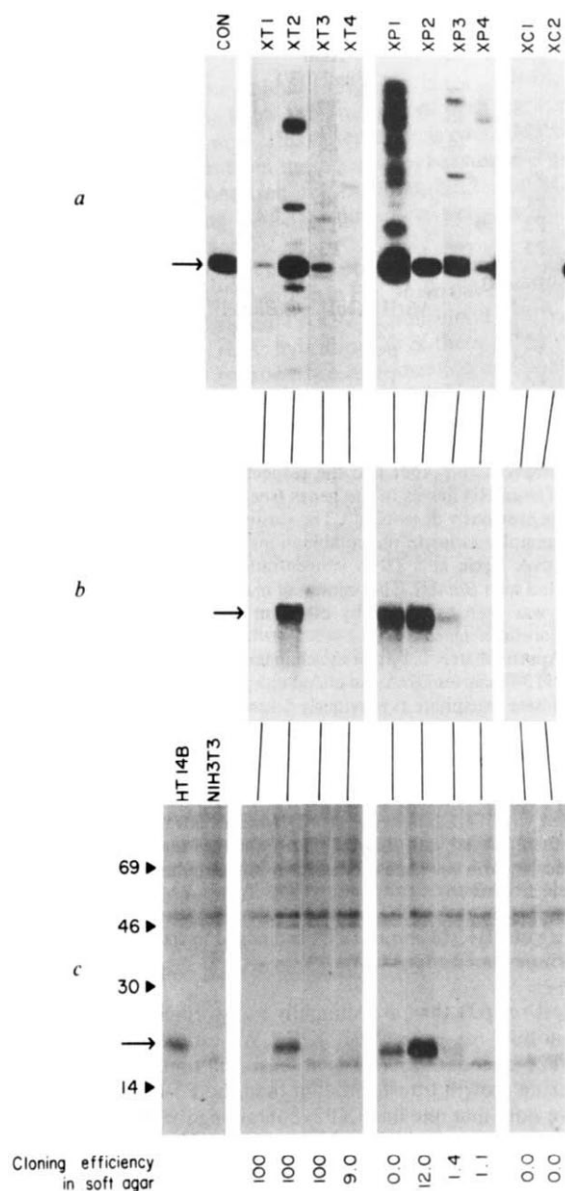


Fig. 2 Expression of normal and transforming H-ras-1 genes after co-transfer into NIH 3T3 cells. NIH 3T3 cells containing the human H-ras-1 gene cloned from T24 (XT1...XT4) or from placental DNA (XP1...XP4) were obtained by co-transfer¹⁸ after selection for expression of the pSV2gpt vector¹¹ added in the presence of an excess of the purified *Bam*HI fragments containing the H-ras-1 gene. Cells were analysed for H-ras-1 gene sequences (a), expression of H-ras-1 transcripts (b), expression of the p21 ras gene product (c), and cloning efficiency in soft agar (bottom). XC1 and XC2 are NIH 3T3 cells containing the pSV2gpt vector but no human H-ras-1 gene. HT14B are NIH 3T3 cells transformed with unintegrated Harvey sarcoma viral DNA. a, 6 μ g DNA from the indicated cell lines were cut with *Sst*I, electrophoresed in 1.0% agarose gels, filter-blotted according to Southern¹⁹ and filters hybridized with 2.9-kb *Sst*I fragment purified from the human H-ras-1 gene (see Fig. 1) and nick translated with ³²P (ref. 20) to a specific activity of 3×10^8 cpm μ g⁻¹. Filter hybridization and washing conditions were at 72 °C under stringent conditions as previously described⁴. Filters were autoradiographed at -70 °C for 15 h with XR5 film and intensifying screens. Lane labelled CON represents 100 ng of purified 2.9-kb *Sst*I fragment. The presence of the 2.9-kb *Sst*I fragment (arrow) indicates the presence of at least one functional copy of the H-ras-1 gene. Other hybridizing fragments represent integration events in which one or both of the *Sst*I sites flanking the H-ras-1 gene were destroyed. b, Cytoplasmic RNA from the indicated cell line was prepared²¹ and 20 μ g were electrophoresed in 1.0% agarose formaldehyde gels as previously described¹⁰, filter blotted and hybridized according to Thomas²² except that dextran sulphate was omitted. The hybridization probe was as described above. The arrow indicates the position of the 1.1–1.2-kb H-ras mRNA. c, Indicated cells were labelled for 6 h in methionine-free Dulbecco's medium containing 5% dialysed serum and 100 μ Ci ml⁻¹, 80 Ci mmol⁻¹ ³⁵S-methionine. Washed cells were lysed in 1% Triton, 0.5% deoxycholate in phosphate-buffered saline containing 2 μ g aprotinin (Sigma) and 1 mM phenylmethylsulphonylfluoride, and centrifuged at 100,000g for 12 h. Supernatants were preabsorbed with goat anti-rat IgG and *Staphylococcus aureus* protein A. Immunoprecipitation was performed for 5 h at 4 °C with anti-v-H-ras p21 monoclonal antibody, the generous gift of E. Scolnick and M. Furth. After addition of goat anti-rat IgG, immune complexes were absorbed to protein A. Pellets were boiled in SDS sample buffer²³ and analysed by SDS-polyacrylamide gel electrophoresis by the method of Blattler *et al.*²⁴. Proteins were visualized after fluorography by exposure for 30 h to Kodak XR5 film. The arrow marks the position of migration of v-H-ras p21. Arrowheads are size markers (MW $\times 10^{-3}$) from ¹⁴C-labelled standards (bovine serum albumin, ovalbumin, carbonic anhydrase and lysozyme; Amersham). Cloning efficiency in soft agar was determined by plating 10^5 cells in 5-cm dishes containing 0.35% Difco agar and Dulbecco's medium with 10% calf serum. 14 days later, numbers of colonies with greater than 10 cells were tabulated for each cell line by examination of six 2-mm diameter microscopic fields.

growth transformation mediated by low levels of an altered *ras* gene product.

We and others have shown transforming *ras* genes are present in many different human tumour cell lines^{6–9}. We speculate that the activation of *ras* genes in these other cell lines is the consequence of somatic mutations in coding regions. If such mutations do occur in human tumour cells, the altered protein products might be immunogenic in their human hosts. The presence, therefore, of antibodies against *ras* proteins in humans may be diagnostic for certain forms of cancer.

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Note added in proof: Substantially similar findings were recently reported for the transforming genes of T24 (ref. 26) and EJ (ref. 27), another bladder carcinoma cell line which we believe

has arisen from T24 (M.W., M.G., J. Fogh, N. Dracopoli and M. Pollack, unpublished data). Most importantly, the predicted amino acid sequences are all in agreement. We note the following differences in the critical 245-bp *Msp*II/*Xba*I fragment: (1) a *Msp*II cleavage site 9 bases 5' to the ATG start codon is predicted and present in the genes we have sequenced, but not predicted by the published sequences of the other groups. (2) A *Nar*I cleavage site 104 bases 3' to the ATG start codon is predicted and present in the genes we have sequenced, but not found in the sequence published by Tabin *et al.*²⁷. (3) An altered histidine codon is not observed for the normal gene sequenced by the other groups.

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A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia

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The transforming genes of oncogenic retroviruses are homologous to a group of evolutionary conserved cellular *onc* genes¹. The human cellular homologue (*c-abl*) of the transforming sequence of Abelson murine leukaemia virus (A-MuLV) was recently shown² to be located on chromosome 9. The long arm of this chromosome is involved in a specific translocation with chromosome 22, the Philadelphia translocation (Ph¹), t(9;22)(q34,q11), which occurs in patients with chronic myelocytic leukaemia (CML)^{3–5}. Here we investigate whether the *c-abl* gene is included in this translocation. Using *c-abl* and *v-abl* hybridization probes on blots of somatic cell hybrids, positive hybridization is found when the 22q[−] (the Philadelphia chromosome), and not the 9q⁺ derivative of the translocation, is present in the cell hybrids. From this we conclude that in CML, *c-abl* sequences are translocated from chromosome 9 to chromosome 22q[−]. This finding is a direct demonstration of a reciprocal exchange between the two chromosomes⁶ and suggests a role for the *c-abl* gene in the generation of CML.

The human *c-abl* sequences represent a cellular homologue of the transforming component of A-MuLV. This retrovirus is a recombinant between Moloney MuLV and mouse cellular *c-abl* sequences⁷ and induces lymphoid tumours on *in vivo* inoculation of the mouse^{8,9}. The major A-MuLV translational product has been identified as a poly-protein, P120^{gag-abl}, consisting of amino-terminal structural proteins encoded by the M-MuLV *gag* gene, linked to an acquired cellular sequence encoded carboxy-terminal component^{10,11}. This protein is one of several virus-encoded transforming proteins with tyrosine-specific protein kinase activity^{12–15}. Similar oncogenic sequences of Harvey and Kirsten sarcoma virus are homologous to transforming sequences (c-Ha-ras, c-Ka-ras) isolated from human bladder and lung carcinoma cell lines^{16–18}. Both these sequences induce transformation of mouse NIH 3T3 cells after transfection, establishing that the human genes have potential transforming activity. Recently, the human *c-abl* gene has been cloned in cosmids¹⁹. Using *v-abl* DNA as a probe, several clones containing overlapping sequences representing the entire *c-abl* gene were isolated from a human lung carcinoma cosmid library. The restriction enzyme map of the human *v-abl* cellular homologue, presented in Fig. 1, identifies areas of the gene which hybridize to *v-abl* sequences. The gene is distributed over a region of 40 kilobases (kb) of human DNA and contains multiple intervening sequences. On transfection of Rat-2 cells with the *c-abl* cosmids, no transforming activity was detected, not unexpectedly, as none of the cosmid clones tested contained the entire *c-abl* gene¹⁹.

By Southern blot analysis of a series of somatic cell hybrids, the human *c-abl* gene has been localized on chromosome 9². This finding is of interest because of the involvement of the long arm of chromosome 22 (band 22q11) in a specific translocation with the long arm of chromosome 9 (band 9q34), the Philadelphia translocation (Ph¹), occurring in human CML^{3,4}. The abnormal chromosomes are designated 9q⁺ and 22q[−]; of these, the 22q[−] chromosome is observed in 92% of CML cases⁵. We investigated the chromosomal location of the human *c-abl*

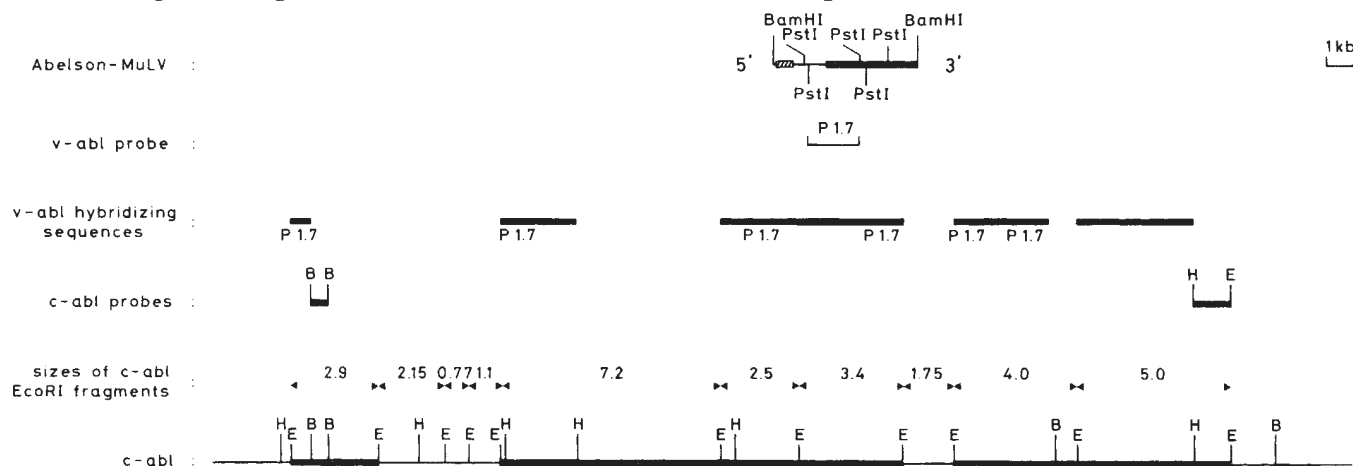


Fig. 1 Restriction enzyme map of the human *c-abl* region¹⁹. The upper line of the figure shows the *Bam*HI subclone of A-MuLV; the hatched box presents the long terminal repeat, the solid bar the acquired cellular sequences. Directly beneath the A-MuLV genome, a subgenomic *Pst*I 1.7-kb fragment, used as a probe in this study, is shown. Human *c-abl* DNA restriction fragments homologous to *v-abl* sequences are indicated as black boxes and those that show homology to the 1.7-kb *Pst*I *v-abl* fragments are designated by P 1.7. The third line shows the human *c-abl* 0.6-kb *Bam*HI and 2.2-kb *Hind*III–*Eco*RI probes, which hybridize to 5' and 3' *c-abl* *Eco*RI fragments, respectively. The sizes of all *Eco*RI *c-abl* fragments are indicated on the fourth line. The bottom line represents the restriction enzyme map of the human *c-abl* gene. Restriction enzymes include *Bam*HI (B), *Hind*III (H) and *Eco*RI (E). A more detailed characterization of the human *c-abl* locus will be published elsewhere¹⁹.

Table 1 Human chromosome content of human-mouse and human-Chinese hamster somatic cell hybrids

Hybrid	Human chromosomes																						Human isoenzyme AKI	Ref.				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22			X	Y	9q ⁺	22q ⁻
PgMe-25NU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	26
10CB-23B	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	6
14CB-21A	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+	20
1CB-17a NU	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-	+	-	+	-	-	-	-	-	+	6
WESP-2A	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	x

The origin and details of the initial characterization of the somatic cell hybrids are described in the references listed in the last column. PgMe-25NU and WESP-2A are hybrids obtained from fusions with mouse Pg19 and WEHI-3B cells, respectively. Chinese hamster cell line E36 was used to produce hybrid clones 10CB-23B and 14CB-21A, while Chinese hamster cell line a3 was used to obtain 1CB-17aNU. Chromosome analysis was done using reverse (R) banding with acridine orange, after heat denaturation. At least 16 metaphases were analysed per cell line. The presence of human AKI activity was assayed by cellulose acetate (Cellologel) electrophoresis²⁷. This test is inconclusive for the WESP-2A cell line (×), because the expression of AKI was found to be repressed in hybrids derived from fusion with WEHI-3B cells (A.H.M., G.v.K., unpublished results). Chromosome and isoenzyme analyses²² were performed on the same batches of hybrid cells that were used for the isolation of DNA.

gene in cases of CML, where the Philadelphia translocation is present. Southern blot analyses with *c-abl* and *v-abl* probes were performed on *Eco*RI-digested DNAs from somatic cell hybrids segregating the 9q⁺ and 22q⁻ chromosomes.

The cell hybrids used here contain a full complement of mouse or Chinese hamster chromosomes and a limited number of human chromosomes. The hybrid cell lines have been obtained by fusion of cells from mouse (Pg 19 and WEHI-3B) or Chinese hamster (E36 and a3) origin with leukocytes from different CML patients and from a normal donor^{6,20}. The human chromosome content of these cells is summarized in Table 1 and is based on chromosome analysis. In addition, the hybrid cells were tested for the expression of human adenylate kinase-1 (AK1) enzyme activity, a marker localized proximal in band 9q34 (ref. 21). This latter test was necessary to exclude the

possibility of hidden (broken or rearranged) chromosome 9 fragments in the 22 and 22q⁻ cell lines.

Detection of the human *c-abl* restriction fragments in hybrid cell DNAs is often inconclusive using *v-abl* probes, because the human sequences are present in submolar amounts (20–50%) and also because many of the human *c-abl* restriction fragments electrophorese in close proximity with strongly hybridizing mouse or Chinese hamster fragments. To obtain molecular probes with specificity for human *c-abl* sequences, two restriction fragments were isolated from subclones of *c-abl*-containing cosmids, with homology to the presumptive 5' and 3' proximal *Eco*RI fragments of *c-abl*. These are 2.9 and 5.0 kb, respectively, in size (Fig. 1). After hybridization and washing to high stringency (0.1 × SSC)¹², the 5'-terminal 0.6-kb *Bam*HI probe and the 3'-terminal 1.1-kb *Hind*III-*Eco*RI probe cross-

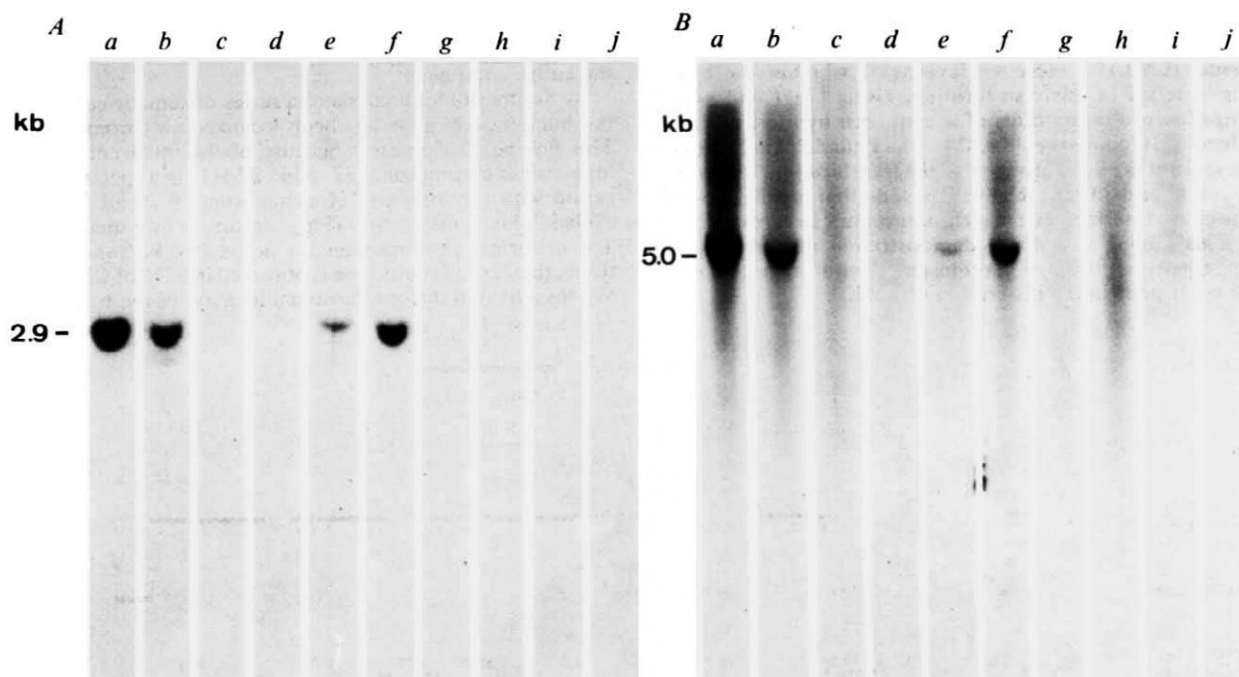


Fig. 2 Localization of human *c-abl* sequences on the Philadelphia chromosome, using hybrid cell lines and human *c-abl* probes. **A**, detection of the human 5' end 2.9-kb *Eco*RI *c-abl* fragment in DNA from *a*, human placenta; *b*, 10CB-23B (chromosome 9); *c*, PgMe-25NU (chromosome 22q); *d*, 14CB-21A (chromosome 9q⁺); *e*, 1CN-17aNU (chromosome 22q⁻); *f*, WESP-2A (chromosome 22q⁻); *g*, mouse Pg19; *h*, Chinese hamster E36; *i*, mouse WEHI-3B; *j*, Chinese hamster a3. **B**, detection of the 3'-end 5.0-kb *Eco*RI *c-abl* fragment in DNAs as indicated in **A** (*a*–*j*). The derivations of all these cell lines and their complements of human chromosomes are summarized in Table 1.

Methods: All cell lines used in this experiment were grown in large batches (10^7 – 10^8 cells) and DNA was prepared as described by Jeffreys and Flavell²⁸. *Eco*RI-restricted DNAs (10 µg per lane) from human placenta, hybrid cell lines, mouse and Chinese hamster fusion partners were electrophoresed on 0.7% agarose gels. *Hind*III and *Hind*III-*Eco*RI-digested λ DNAs were included as molecular weight markers (not shown). After blotting to nitrocellulose, the filters were hybridized to the 0.6-kb *Bam*HI *c-abl* (**A**) or 1.1-kb *Hind*III-*Eco*RI *c-abl* (**B**) restriction fragments described in Fig. 1. Hybridization and washing procedures (to 0.1 × SSC at 65 °C) were carried out according to the method of Bernards and Flavell²³.

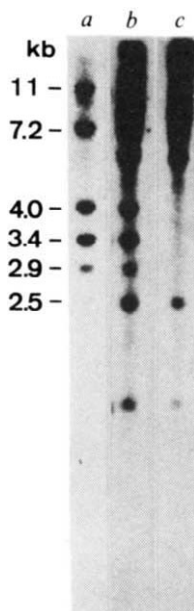


Fig. 3 Localization of human *c-abl* sequences on the Philadelphia chromosome, using a 22q⁻ somatic cell hybrid and a *v-abl* probe. *Eco*RI-digested DNAs (10 µg) from human placenta (a), hybrid WESP-2A (b) and mouse WEHI-3B cells (c) were hybridized with the 1.7-kb *Pst* *v-abl* fragment (Fig. 1), as described in Fig. 2 legend. After hybridization, the filters were washed to 1 × SSC at 65 °C. Molecular weights of human *c-abl* fragments were deduced from co-electrophoresed *Hind*III and *Hind*III-*Eco*RI-digested λ DNA markers.

hybridize to a very low extent with mouse or hamster *c-abl* sequences. Figure 2A shows an example of a hybridization experiment with the 0.6-kb *Bam*HI probe. This Southern blot illustrates hybridization of *Eco*RI-restricted DNAs of hybrid cell lines containing chromosomes 22, 9, 9q⁺ or 22q⁻. As controls, hybridization of the probe with human placenta DNA and DNA from the mouse and Chinese hamster fusion partners is shown. It is clear that the 2.9-kb *Eco*RI fragment, detected in human placenta DNA, is also present in the lanes containing DNA from the hybrid cell lines 10 CB-23B (chromosome 9), 1CB-17a NU and WESP-2A (both containing chromosome 22q⁻). The band is not detected in lanes containing DNA from PgMe-25Nu (chromosome 22), 14CB-21A (chromosome 9q⁺), Pg19 and WEHI-3B (mouse controls) or E36 and a3 (Chinese hamster controls). Analogous results are obtained when the same *Eco*RI-digested DNAs are hybridized to the 3'-terminal 1.1-kb *Hind*III-*Eco*RI probe (Fig. 2B). The 5.0-kb *Eco*RI fragment is detected only in DNA from human placenta and from hybrid cell lines containing chromosome 22q⁻ or 9.

The above results show that both the 5' and 3' ends of the *c-abl* gene are translocated to chromosome 22q⁻. Because all other *c-abl* *Eco*RI fragments, which hybridize to *v-abl* sequences, are flanked by the 2.9-kb and 5.0-kb *Eco*RI fragments, it seems highly probable that these fragments are also included in the translocation to the Philadelphia chromosome. To test this possibility directly, hybridization was performed using a 1.7-kb *Pst* *v-abl* probe (Fig. 1). Because of the problems with *v-abl* probes indicated above, only WESP-2A, the hybrid containing the most 22q⁻ sequences (50% of the molar amount), was tested. As shown in Fig. 3, the viral probe detects human *Eco*RI *c-abl* fragments of 11, 7.2, 4.0, 3.4, 2.9 and 2.5 kb (weakly). Of these fragments, the 11-kb band has been shown to map outside the main human *c-abl* locus¹⁹ and will not be considered here. The human 2.9-, 3.4-, and 4.0-kb *c-abl* fragments are readily detected in the WESP-2A DNA. In contrast, the 7.2-kb *Eco*RI fragment can only be seen in a short exposure of this filter (not shown), due to spill-over of radiation from strongly hybridizing mouse *c-abl* fragments in this area. The

2.5-kb *Eco*RI human *c-abl* fragment co-migrates with a mouse fragment of similar size and thus cannot be identified in this analysis.

The hybrid cell lines containing the 9q⁺ and 22q⁻ chromosomes examined in the present study, were obtained from fusion experiments with CML cells from three different individuals. Therefore, we conclude that in the Philadelphia translocation a fragment of chromosome 9 is translocated to chromosome 22q⁻ and that this fragment includes the human *c-abl* sequences. This finding establishes that the translocation is reciprocal, a general assumption which is now demonstrated unequivocally. Moreover, the data map the human *c-abl* sequences distal to AK1 (not translocated to 22q⁻, 6, 20) on chromosome 9. The most interesting aspect is that it raises the possibility of involvement of the human *c-abl* gene in the generation of CML.

In principle, the chromosomal translocation associated with CML could lead to elevated levels of *c-abl* expression which, by analogy to the *c-Ha-ras* gene in bladder carcinoma, would induce malignant transformation²⁴. Elevated levels of *c-abl* expression could be the result of coupling of the gene to an enhancer sequence present on chromosome 22 or, alternatively, the gene could be linked to a strong promoter of another gene. To test these possibilities, we have initiated studies to clone the *c-abl* gene from the 22q⁻ chromosome using WESP-2A DNA and a cosmid vector system. Finally, it is of interest that in some CML patients variant Ph¹ translocations are observed, in which the participation of chromosome 9 cannot be detected by classical cytogenetic analysis^{3,5}. In another group of CML patients the Ph¹ translocation appears to be completely absent²⁵. We are now investigating whether the *c-abl* gene is translocated to chromosome 22 in these cases also.

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Abnormal RNA processing due to the exon mutation of β^E -globin gene

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As is typical of all β -thalassaemias¹, the erythroid cells of individuals with the variant haemoglobin E ($\alpha_2\beta_2^{26\text{Glu}\rightarrow\text{Lys}}$) exhibit a quantitative deficiency in their content of β -globin (in this case β^E -globin) and its messenger RNA^{2,3}. To determine the molecular basis of this phenotype, we have investigated the structure and expression of cloned β^E -globin genes. We report here that the complete nucleotide sequence of a β^E -gene revealed the expected GAG→AAG change in codon 26 but no other mutations. Expression of β^E -globin genes introduced into HeLa cells revealed two abnormalities of RNA processing: slow excision of intervening sequence-1 (IVS-1) and alternative splicing into exon-1 at a cryptic donor sequence within which the codon 26 nucleotide substitution resides. These results demonstrate a disturbance in the expression of the β^E -gene attributable solely to the exon mutation—a novel mechanism for gene dysfunction.

We first examined the nucleotide sequences of β^E -globin genes to determine whether there were two mutations, one in codon 26 and another elsewhere in the gene causing a reduction of mRNA level, or only a single mutation. We studied two different cloned β^E -globin genes that were distinguishable by their intragenic DNA polymorphisms⁴. Underproduction of β^E -globin in individuals bearing either of these two gene types suggests a close relationship between the protein/mRNA deficiency and the exon mutation. The complete DNA sequence of one of the β^E -globin genes (β_1^E), determined by the method of Maxam and Gilbert⁵, revealed the expected codon 26 change (GAG→AAG) and four previously identified intragenic polymorphisms⁴, but no other substitutions. Partial DNA sequencing of the other β^E -gene (β_2^E) also showed the codon 26 change and a single intragenic polymorphism⁴. Unless a common extragenic mutation were associated with these apparently independently derived β^E -genes, these sequence data indicate that the codon 26 change must be directly related to the underproduction of β^E -mRNA.

The expression of the cloned β^E -globin genes linked to Simian virus 40 (SV40) sequences in a miniplasmid⁶ was studied after their introduction into HeLa cells. In this transient expression system, transcripts of the normal β -globin gene are efficiently synthesized and appropriately processed⁶⁻⁸. In all experiments we compared transcripts of the two β^E -globin genes with those of a normal gene. Cells were collected either 24 or 48 h after transfection and RNA was analysed by Northern blot analysis⁹ and S₁ nuclease mapping¹⁰ using a variety of labelled DNA probes. Both β^E -globin genes directed synthesis of near normal amounts of 9S β -globin RNA (not shown). S₁ nuclease mapping of total cell, or fractionated cytoplasmic and nuclear, RNAs revealed two striking abnormalities of β^E -RNA processing. First, the steady-state level of RNA containing IVS-1 sequences was increased (Fig. 1). Second, a fraction of β^E -transcripts was alternatively spliced (Figs 1, 2); in our hybridization conditions this abnormal product represented 5–8% of the total, a value confirmed by primer extension analysis using an exon-2 fragment.

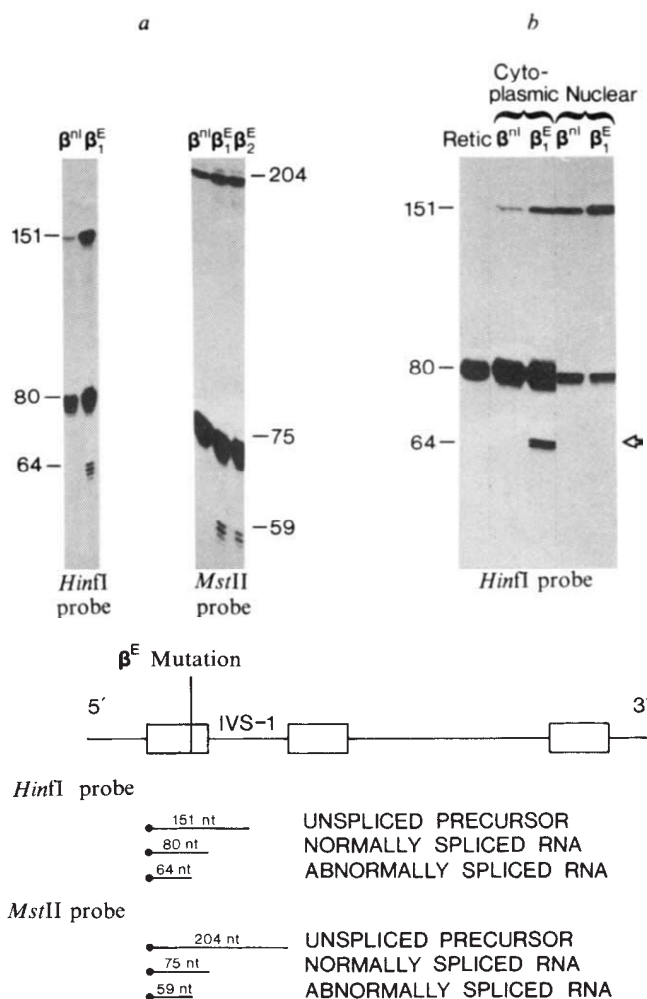


Fig. 1 Slow and alternative processing of β^E -RNA transcripts. β^E -globin genes of frameworks 1 (β_1^E) and 3 (β_3^E)^{4,16} were cloned in bacteriophage Charon 28 as described previously and subcloned as a 3.7-kilobase (kb) $BglII/PstI$ fragment in the miniplasmid π SV40plac⁶. These β^E -gene subclones and a normal (nl) β -globin gene oriented identically in the same expression vector, were transfected to subconfluent HeLa cells as described by Treisman *et al.*⁶. Total cellular RNAs (a) were isolated by repeated guanidine HCl extractions and phenol extraction at 24 h (left) and 48 h (right) after transfection. Cytoplasmic and nuclear RNA fractions (b) were prepared from cells 24 h after transfection as described by Favaloro *et al.*¹⁷. The 3'-end-labelled, single-stranded DNA probes indicated were prepared from β^E -globin gene DNA by filling in 5'-overhangs with ³²P-dXTPs (each at 600 Ci mmol⁻¹) using the Klenow function of *Escherichia coli* DNA polymerase I and isolation of the anti-sense strand⁵; 25 μ g total or cytoplasmic and 10 μ g nuclear RNAs plus DNA probe were denatured in hybridization buffer¹⁷, incubated at 37 °C for 20 h, and then treated with 1,000 U ml⁻¹ S₁ nuclease (Sigma) at 37 °C for 40 min. Reticulocyte globin mRNA (6.5 ng) was assayed in parallel as a control. Protected DNAs were electrophoresed in urea-8% acrylamide gels at 2,000 V for 2 h; the gels were then exposed to X-ray film with intensification. Hybridization of the RNA and DNA probe at 51 °C rather than 37 °C led to a marked reduction in protected fragments 59 or 64 nucleotides (nt) long due to instability of short RNA-DNA hybrids at this temperature in formamide buffer (data not shown). Identical S₁ nuclease mapping results (shown above) were obtained with probes prepared from both normal and β^E -gene DNA. Note the presence of alternatively or abnormally spliced RNA in the β^E -RNA samples and the 3–5-fold increase in unspliced material relative to normal. The 64-nucleotide DNA fragment was clearly visible in the nuclear sample on the right on the original autoradiogram, as indicated by the open arrow.

Alignment of the novel protected DNA fragment observed in these S₁ mapping experiments with sequencing tracts of the probe assigned the alternative splice site to an exon-1 donor-like^{11,12} sequence, GTG⁴GTAAGG, that spans the codon 26 mutation itself (Fig. 2). The normal sequence of this portion of exon-1, GTGGTGAGG, resembles donor (or 5') splice sites^{11,12}. This site is not used in splicing normal gene transcripts

(Fig. 1), but is employed in processing RNA transcripts of three different β -thalassaemic genes having substitutions in or near the IVS-1 splice junction (R. A. Treisman *et al.*, in preparation). Activation of this cryptic donor site by the β^E mutation is selective, however, in that additional cryptic sites used in these latter cases are not used to process β^E -transcripts. The cryptic splicing site generated by the codon 26 mutation apparently has sufficiently enhanced affinity with the splicing apparatus to allow it to compete with the normal donor site and retard IVS-1 excision. Additional S_1 nuclease mapping studies (not shown) revealed use of the normal IVS-1 acceptor and IVS-2 donor and acceptor sites in processing β^E -transcripts. The alternatively spliced β^E -RNA cannot encode normal globin due to loss of a portion of the exon as well as production of a downstream in-phase terminator in the processed product. Thus, the HeLa cell experiments demonstrated that β^E -precursor RNA is processed both slowly and alternatively (Fig. 3).

The *in vivo* situation in erythroid cells may be more complex than that in HeLa cells due to potential quantitative differences in mRNA metabolism. In total bone marrow RNA of one haemoglobin E homozygote, we have found little, if any, alternatively spliced steady-state β -RNA (not shown). However, in other thalassaemias where alternative splicing occurs as the predominant or only mode of processing of mutant globin transcripts in heterologous cells, alternatively spliced RNAs are detectable at comparatively low levels *in vivo*, probably due to their instability in erythroid cells^{6,8,13,14}. Therefore, the apparent absence of such a species from steady-state β^E -bone marrow RNA does not necessarily signify a different processing pattern in erythroid cells, but does prevent full assessment of the contribution of abnormal processing to underproduction of β^E -mRNA. Previously, cytoplasmic instability of β^E -mRNA in erythroid cells has been proposed as another basis for the thalassaemic phenotype¹⁵. We have observed variable and extensive fragmentation of β^E -mRNA isolated from reticulocytes, but not bone marrow cells, that is consistent with these previous data. However, as most globin is produced in marrow rather than peripheral cells, the contribution of peripheral cytoplasmic instability of β^E -mRNA to decreased β^E -globin synthesis is also unclear. The difficulties in reconciling the results of the surrogate genetic experiments in HeLa cells with those in erythroid cells may largely reflect the rather mild deficit in expression of the β^E -globin gene.

The deleterious effect of a nucleotide substitution in an exon on the rate as well as the pattern of processing of a globin RNA

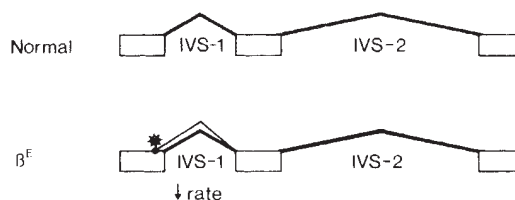


Fig. 3 Splicing patterns of normal and β^E transcripts in HeLa cells. Normal RNA is spliced exclusively from the ends of the exons as depicted. IVS-1 of β^E -RNA transcripts is removed slowly and alternative splicing into exon-1 near the β^E -mutation (asterisk) occurs. We have used the compilations of normal sequences of donor sites reported by Chambon¹¹ and Mount¹² to estimate the relative affinities of potential splicing sites. The relevant sequences seem to include three nucleotides preceding the invariant GT and four nucleotides thereafter. We have merely summed the number of genes reported in each compilation with a specific nucleotide at each of these positions for the sequences of the normal 5'-IVS-1 splice site, and the normal and β^E cryptic sites; using the listing of Chambon¹¹, the sums are 489, 448, and 468, respectively. A 'score' of 583 is the maximum possible. From the listing of Mount¹², values of 758, 577, and 622 are obtained with a maximum of 913. Overall, the codon 26 substitution makes the cryptic site of the normal more favourable as a splice site, although still not as strong as the normal site.

precursor is a newly recognized mechanism of gene dysfunction. The cryptic splice site enhanced by the β^E mutation is also mutated in two other globin genes associated with a thalassaemic phenotype. First, in a β^T -thalassaemic gene, a silent T $\rightarrow$ A substitution in codon 24 has recently been discovered and shown to affect the processing of RNA transcripts in heterologous cells (A. Nienhuis, personal communication). Second, the predicted DNA sequence GTGGTGAGT in exon-1 of a newly described and underproduced β -chain variant, Knossos ($\beta^{27\text{Ala} \rightarrow \text{Ser}}$) (D. Loukopoulou, personal communication) seems as favourable as an authentic donor splice site. We anticipate that transcripts of this β -chain variant will also be alternatively spliced at this cryptic site. Taken together, the underproduction of β -globin from three different genes having mutations within the same cryptic splice site strongly suggests that the alternative RNA splicing we have described is of physiological significance. These principles may easily be expanded to account for underproduction of many gene products associated with inherited disease in man.

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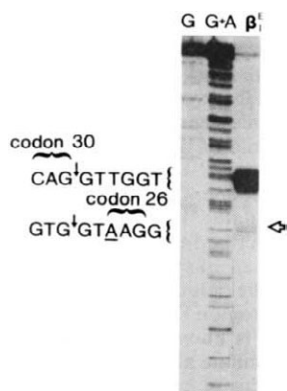


Fig. 2 Location of normal and alternative splices in the β^E gene sequence. Cytoplasmic RNA of HeLa cells transfected with the β^E gene was analysed by S_1 nuclease mapping with the 3'-labelled *Hinf*I probe. G and G+A sequencing reactions⁵ of the probe were electrophoresed in parallel lanes. The corresponding sequence of the mRNA strand is shown on the left with the splicing sites indicated by the GT rule¹¹. The underlined A in codon 26 is the mutated nucleotide in the β^E -mRNA.

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The deletion in a type of $\delta^0\text{-}\beta^0$ -thalassaemia begins in an inverted *AluI* repeat

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In hereditary persistence of fetal haemoglobin (HPFH) and $\delta^0\text{-}\beta^0$ -thalassaemia, increased levels of fetal haemoglobin (HbF) are found in adult individuals. HbF production is particularly noticeable in the former condition in which HbF levels of 20–40% are found in heterozygous patients, as opposed to 5–20% in $\delta^0\text{-}\beta^0$ -thalassaemia¹. In a minority of these cases, no obvious abnormalities have been found in the globin gene region by DNA mapping^{2–6}, indicating that small deletions or perhaps even point mutations in critical DNA sequences in the globin gene cluster may be responsible for these conditions. However, most cases of HPFH and $\delta^0\text{-}\beta^0$ -thalassaemia are associated with extensive deletions in the globin gene cluster^{2–4,7–14}. Genetic data¹⁵ and gene mapping investigations^{2,4,10} provided some evidence for the location of a regulatory area, whose deletion results in continuing activity of γ -globin genes in adults, in a DNA region between the γ - and δ -globin genes, possibly 3–4 kilobases (kb) 5' to the δ gene. The precise nature of these sequences is of great interest, because it might help elucidate the molecular mechanisms regulating globin gene expression during development. Recently, Jagadeeswaran *et al.* cloned¹⁶ the DNA encompassing the region of a gene deletion in a type of HPFH^{2,10} and showed¹⁶ that the 5' end point of the deletion lies in the middle of an *AluI* repetitive DNA sequence^{17,18}. We have now cloned the corresponding region from the DNA of a $\delta^0\text{-}\beta^0$ -thalassaemia patient⁴ and we report here that the deletion ends in a different *AluI* sequence, ≈ 700 nucleotides 3' to the *AluI* repeat involved in the HPFH deletion, and in the opposite orientation.

The isolation and characterization of the $\delta^0\text{-}\beta^0$ -thalassaemic DNA clone will be described elsewhere (B.G. and S.O., in preparation). Figure 1 shows a restriction endonuclease map of the human non- α -globin gene cluster in the region of the $\delta^0\text{-}\beta^0$ deletion end point. This occurs ≈ 3.8 kb 5' to the δ -globin gene. Previous analyses^{19,20} demonstrated the existence in this area of a pair of repetitive DNA sequences, belonging to the *AluI* family, pointing in opposite directions and separated by ≈ 700 base pairs (bp) of non-repetitive DNA.

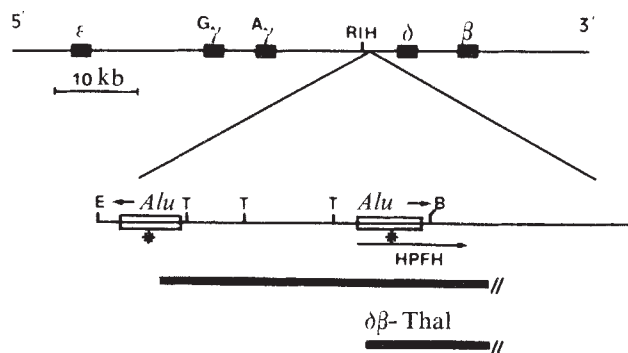


Fig. 1 Schematic representation of the human non- α -globin gene cluster (top) and expanded view of the normal region¹⁹ where the breakpoints occur in HPFH¹⁶ and in $\delta^0\text{-}\beta^0$ -thalassaemia (bottom). RIH is a unique sequence DNA fragment¹⁰. Black boxes show the deletions, short arrows indicate the polarity of the *AluI* repeats, and the long arrow the transcriptional unit²⁰. Asterisks mark the position of sequences homologous to origins of replication of mammalian viruses¹⁷. E, *EcoRI* site; T, *TaqI* site; B, *BglII* site.

-100	TTTGTCCCTA	TCTATTAATC	ACCACTCTTA	CTGCCAGTC	AGGTCTCAT
	TTTGTCCCTA	TCTATTAATC	ACCACTCTTA	CTGCCAGTC	AGGTCTCAT
-50	TUTTTCTCTA	ACAAGAGTAG	ATGCTATTCT	TTCACCTTTT	AGACCTTATC
	TUTTTCTCTA	ACAAGAGTAG	ATGCTATTCT	TTCACCTTTT	AGACCTTATC
1	TGGCTGGATG	CGGTGCTCTA	GGCTTGTAAA	ECCAGCACTT	TGGAGGCCA
	TGGCTGGAGG	AGCTGGCTCC	GGCTTGTGCC	AGCCCAAAA	GGGCTTCCA
51	AGGCAGGCAG	ATCACTTGA	GTCAAGGATT	CAGACCAAGC	CTHACCAACA
	***	*****	*****	*****	*****
	CAGTGCATG	GGGGCTGAA	GGGCTCTCTA	AGTGGGCCA	ACTGGACCA
					$\delta^0\beta^0$ Thal

Fig. 2 Comparison of the normal DNA sequence (upper line) with that of DNA across the 5' end point of the deletion in $\delta^0\text{-}\beta^0$ -thalassaemia (lower line). The normal *AluI* sequence (from nucleotide 1 onwards) is from ref. 20. Asterisks show where the nucleotides of the two sequences differ. Solid lines overlie nucleotides corresponding to *AluI* DNA. Arrows underlie consensus sequences, possibly involved in specifying transcriptional initiation sites^{20,21}.

Fine restriction mapping of our clone (S.O. and B.G., in preparation) demonstrates normal bands from the *EcoRI* site to the most 3' *TaqI* site; we therefore sequenced the $\delta^0\text{-}\beta^0$ -thalassaemic DNA 3' to this *TaqI* site and compared it with the normal DNA sequence (Fig. 2). The two sequences show perfect homology in a 100-nucleotide long fragment. This is followed by an eight-nucleotide sequence in $\delta^0\text{-}\beta^0$ -thalassaemic DNA that is identical to the beginning of the normal 3' *AluI* repeat; beyond this point the $\delta^0\text{-}\beta^0$ -thalassaemic DNA continues into a G-rich region having little homology with the normal *AluI* sequence. Thus, the $\delta^0\beta^0$ deletion almost completely removes the 3' *AluI* sequence, leaving intact the 5' repeat and non-repetitive DNA in between. In contrast, in the HPFH type sequenced by Jagadeeswaran *et al.*¹⁶, the entire 3' and part of the 5' *AluI* sequence are absent.

HPFH heterozygotes produce during adult life considerably more HbF than $\delta^0\text{-}\beta^0$ -thalassaemic heterozygotes. According to the model proposed by Huisman *et al.*¹⁵ (see also refs 2, 4, 10), deletion of an unidentified regulatory sequence between the γ - and δ -globin genes is sufficient to cause a high level of persistent activity of γ -globin genes *cis* to the defect. Thus, comparison of the different deletion 5' end points in HPFH¹⁶ and $\delta^0\text{-}\beta^0$ -thalassaemia identifies the 5' *AluI* repeat and/or non-repetitive DNA connecting it to the 3' *AluI* repeat as possible candidates for such a key regulatory role. This region is absent from all deletion-type HPFH reported so far, but is present in $\delta^0\text{-}\beta^0$ -thalassaemias. (The type of $\delta^0\text{-}\beta^0$ -thalassaemia in which expression of the γ gene exclusively is found¹⁰, is the only apparent exception to this rule. Here, the deletion removes both *AluI* repeats, but also extends to the γ gene, thus limiting HbF synthesis; it is probably better considered as a γ - δ - β -thalassaemia.) Although confirmation of the possible regulatory role of this region will require the study of additional HPFH and δ - β -thalassaemias, it is interesting that this region includes sequences for which many different functions have been postulated^{17,18}. Here, we only mention that the 3' *AluI* repeat is transcribed 'in vitro' by DNA polymerase III²⁰. Although the presumed starting site of transcription²⁰ (position 1 in Fig. 2) is intact, the deletion in $\delta^0\text{-}\beta^0$ -thalassaemia removes the internal 'control' region thought to be essential for transcription^{20,21}. As nothing is known of their 'in vivo' transcription, it is premature to speculate about possible roles of the transcription of these sequences in normal and pathological expression of globin genes; however, note that the almost total loss of the 3' *AluI* repeat in this $\delta^0\text{-}\beta^0$ -thalassaemia only slightly changes the phenotype relative to other δ - β -thalassaemias in which this region is intact⁴. Finally, attention should also be given to possible effects of distant sequences 3' to the β -globin gene which are brought by the deletion into direct contact with the *AluI* repeats; these sequences, which differ between $\delta^0\text{-}\beta^0$ -thalassaemic and HPFH DNA (Fig. 2 and ref. 16) are presently

being located (in collaboration with R. Flavell) by mapping studies and their structure is being analysed.

The mechanism generating the deletion is unknown, but some clue to its understanding may be provided by the sequence of the $\delta^0\beta^0$ -thalassaemia clone around the breakpoint. In normal DNA the octamer, TGGCTGGA, at the beginning of the *AluI* sequence is followed, after a five-nucleotide spacer, by a dodecamer, TGGCTCAGGCTT, including a modified version of the former sequence. In $\delta^0\beta^0$ -thalassaemic DNA, the octamer is intact and is followed, after a six-nucleotide spacer obviously different from the normal one, by a dodecamer which differs at only two positions from the normal one. The normal sequence, by misaligning with a slightly different distant sequence, might thus act as a 'hotspot' for non-homologous recombination, leading to the large deletion observed. Such a mechanism was recently demonstrated in a prokaryotic system²². Cloning of the normal counterpart of the distant sequence should allow us to confirm this mechanism for our case.

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Detection of human papillomavirus DNA in anogenital neoplasias

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The presence of papillomaviruses in epithelial-derived cancers from several animal species^{1–3} has led to the speculation that these viruses may also have a pathogenic role in the development of certain human carcinomas, particularly those associated with the anogenital tract⁴. Recently, human papillomavirus (HPV) DNA has been detected in epithelial-derived cancers, both cutaneous^{1,5} and metastatic⁵, from patients exhibiting the rare, chronic flat wart disease, epidermodysplasia verruciformis (EV). Except for patients exhibiting this chronic wart syndrome, the association of HPV genomes with human epithelial cancers

has not been demonstrated⁶. In an attempt to delineate the association and possible involvement of papillomaviruses with human anogenital carcinomas, we have begun an analysis of these cancers for the presence of HPV-specific nucleotide sequences by using highly sensitive hybridization procedures capable of detecting distantly related papillomaviruses at low copy number. Here we demonstrate the presence of HPV DNA in several types of anogenital tumours: Bowenoid papulosis, carcinoma *in situ*, and verrucous carcinoma. These data indicate that HPV can be detected in several types of premalignant and malignant tumours, supporting the contention that this group of viruses may be involved in the development of certain types of human epithelial-derived cancers.

We have used the Southern blot hybridization procedure to search for HPV DNA in three types of anogenital tumours: verrucous carcinoma, which is a form of invasive squamous cell carcinoma; carcinoma *in situ*; and Bowenoid papulosis, which is histologically identical to but biologically distinct from carcinoma *in situ*. Total cellular DNA was extracted from tumour tissue as described previously⁵ and analysed by the Southern blot hybridization technique⁷. The HPV DNA probes used in these analyses included HPV-EV and HPV-5 obtained from patients with epidermodysplasia verruciformis^{5,8}, and HPV-6 obtained from a patient with condylomata accuminata, a form of anogenital wart⁹. The HPV DNAs were molecularly cloned into pBR322 and the recombinant plasmids were labelled with ³²P *in vitro* by nick-translation¹⁰ resulting in specific activities of 5×10^8 c.p.m. μg^{-1} . Because of the apparent lack of homology between different types of HPV in stringent hybridization^{11,12}, conditions of reduced stringency were used in these analyses. In such low stringency conditions, any specific type of HPV will hybridize to any of the other known varieties of

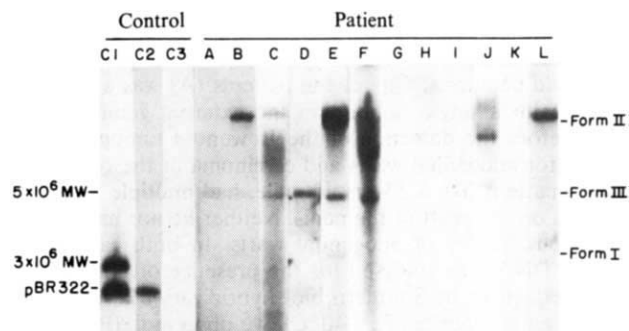


Fig. 1 Detection of HPV DNA in tumour tissue. Total cellular DNA was extracted from tumour tissue as previously described⁵. The DNA was electrophoresed through 1% agarose gels and transferred to nitrocellulose filters by the method of Southern⁷. The filters were dried at 80 °C under vacuum for 2 h and hybridized in conditions of low stringency. The HPV used as probe in these conditions was an HPV species that exhibits homology (~36%) with HPV type 3 in stringent conditions (ref. 8 and M. Green, personal communication) and which for brevity is referred to here as HPV-EV. The filters were pre-hybridized at 37 °C for 24 h in hybridization solution containing 500 $\mu\text{g ml}^{-1}$ depurinated salmon sperm DNA, 0.1% each of bovine serum albumin, Ficoll 400 and polyvinylpyrrolidone, 20 mM sodium phosphate pH 6.8, 1 M sodium chloride, 0.1% SDS, and 30% formamide¹¹. Fresh hybridization solution containing nick-translated ³²P-HPV-EV probe (containing both cloned *Bam*HI fragments (5 ng probe per ml solution; 2.5×10^6 c.p.m. ml^{-1} and 10% dextran sulphate) was added to the filters and incubated at 37 °C for 20 h. Filters were washed three times in $2 \times \text{SSC}$ pH 7.0 (0.3 M sodium chloride, 0.03 M sodium citrate), 0.1% SDS, and 0.1% Na pyrophosphate at room temperature and for an additional 4 h in $3 \times \text{SSC}$ pH 7.0, 0.1% SDS, and 0.1% Na pyrophosphate at 40 °C. Filters were dried and autoradiographed at -70 °C with an intensifying screen. Lane CI contains 10 pg of the 3.0-megadalton fragment of the HPV-EV DNA used as probe that had been cleaved from the pBR322 plasmid with *Bam*HI; also present is the remaining 10 pg of pBR322. Lane C2 contains 100 pg of linear HPV-5 that was prepared from a plasmid composed of HPV-5 and pBR322. The plasmid was cleaved with *Bam*HI and the fragments separated by agarose gel electrophoresis. The linear HPV-5 band was cut out of the gel and the DNA was electroeluted from the agarose as previously described⁵. The lower band is a trace amount of pBR322 that remained in the preparation. Lane C3 contains 10 μg of cellular DNA from a hyperkeratotic lesion. Lanes A–L contain 5–10 μg of cellular DNA from the corresponding patient. The DNA in lane D was cleaved with *Bam*HI, while the remaining cellular DNA samples were not treated with restriction endonucleases.

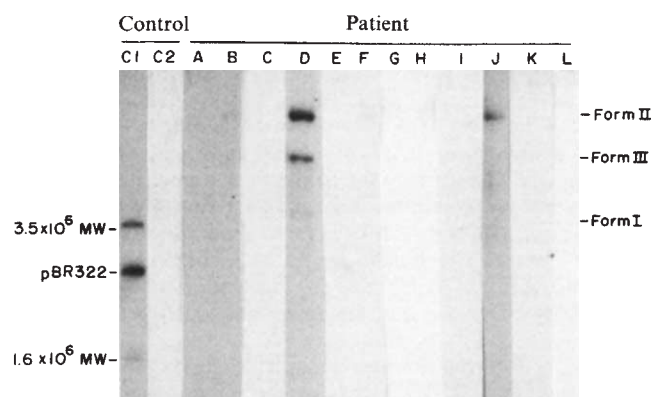


Fig. 2 Hybridization of DNA from tumour tissue with ^{32}P -HPV-6 in stringent conditions. DNA was prepared, electrophoresed and transferred to nitrocellulose as described in Fig. 1 legend. The stringent hybridization conditions were similar to those described in Fig. 1 with the following differences: (1) the pre-hybridization and hybridization solutions contained 50% formamide; (2) the hybridization was carried out at 40 °C; (3) after the room temperature washes, the filters were washed for an additional 4 h in 0.4 × SSC pH 7.0, 0.1% SDS, 0.1% Na pyrophosphate at 50 °C; and (4) the probe used was nick-translated pBR322-HPV-6. Lane C1 contains 10 pg of the 3.5-megadalton fragment and 10 pg of the 1.6-megadalton fragment of HPV-6 DNA; each has been cleaved from the pBR322 plasmid with a *Bam*HI and *Eco*RI double digestion. Lane C2 contains 10 µg of cellular DNA from a hyperkeratotic lesion. Lanes A–L contain 5–10 µg of cellular DNA from the corresponding patient.

HPV by virtue of distantly conserved nucleotide sequences^{12,13}; using these conditions, no hybridization can be detected between DNA from any HPV type and simian virus 40 or BK DNA¹³. The sensitivity of our low stringency hybridization procedure allowed detection of as few as six copies of heterologous HPV genome equivalents per diploid cell (see Fig. 1, lane C2).

Tumour samples were analysed from two patients with Bowenoid papulosis. One of the patients (A) was a 35-yr-old female with a single tumour on the external genitalia. Four years before the detection of the Bowenoid tumour she was treated for anogenital warts and carcinoma of the cervix. The second patient (B), a 33-yr-old male, had multiple Bowenoid tumours on the shaft of the penis. Neither he nor his wife had a previous history of anogenital warts. In both cases, when cellular DNA was analysed for the presence of HPV-related DNA sequences by Southern blot hybridization in conditions of reduced stringency (T_m –40 °C), we observed HPV-specific nucleotide sequences migrating in an identical manner to unintegrated forms of HPV DNA (Fig. 1).

The verrucous carcinoma samples were obtained from a 61-yr-old female (C) with a history of squamous cell carcinoma of the cervix and a verrucous carcinoma on the perineum, and

from a 17-yr-old male (D) with a penile verrucous carcinoma. These tissue samples showed unintegrated HPV DNA in less stringent hybridization conditions (Fig. 1).

Eight vulvar carcinoma *in situ* tissue samples were also obtained for analysis. Four out of eight DNA samples from these patients contained HPV-related DNA when analysed by hybridization in less stringent conditions (see Fig. 1, patients E–L).

Due to the association of HPV-5 DNA with carcinomas from patients exhibiting epidermodysplasia verruciformis^{1,5}, we also tested DNA extracts from many of the verrucous carcinomas and carcinomas *in situ* with labelled HPV-5 in stringent conditions where we could detect as little as 0.1 viral genome copy per cell genome equivalent. With these conditions we were unable to detect any HPV-5-related sequences (Table 1). Moreover, we have tested these tumour DNAs for nucleotide sequences homologous to an HPV (type 6) that is associated with anogenital papillomas¹⁴ and find most of these tumours negative for HPV-6-related sequences (Fig. 2, Table 1).

The viral DNA extracted from each patient's tissue was found to be of typical HPV genome size (~5 megadaltons) with no evidence of any subgenomic fragments, as has been shown in carcinomas from patients with epidermodysplasia verruciformis⁵. Based on the electrophoretic mobility of form I (covalently closed circular), form II (nicked circular) and form III (linear) viral DNA, most of the viral DNA obtained from these tissues seems to be unintegrated although we cannot completely exclude the possibility of integrated viral sequences using this detection technique. Sufficient HPV DNA was not available to perform restriction endonuclease mapping to type the HPV species in these tumours.

From the Southern blot hybridization analysis presented here, it is clear that HPV-specific nucleotide sequences are present in several different anogenital tumour samples. Our ability to detect as few as six copies of heterologous HPV genome equivalents per cell genome using conditions that facilitate hybridization with heterologous HPV types enhanced our ability to detect HPV sequences. This technique is thus more sensitive than that used in a recent study in which carcinomas were analysed for the presence of HPV DNA in stringent hybridization conditions which only detected HPV DNA closely related to that of the radiolabelled HPV DNA probe⁶. Moreover, we have detected HPV DNA in anogenital cancers that were previously thought to lack HPV¹⁴. At present it is unclear what, if any, is the specific involvement of HPV in the development of these anogenital tumours. The 12 patients analysed in this study exhibited three clinical syndromes each characterized by either benign, premalignant or malignant transformation of anogenital skin. The presence of HPV DNA in these tumours suggests but does not implicate a pathogenic

Table 1 Summary of lesions examined and detection of HPV-related sequences

Lesion	Patient	Age	Site	Less stringent (^{32}P -HPV-EV)	Stringent	
					^{32}P -HPV-5	^{32}P -HPV-6
Bowenoid papulosis	A	35	Vulva	+	ND	–
	B	33	Penis	+	ND	+
Verrucous carcinoma	C	61	Vulva	+	–	–
	D	17	Penis	+	–	+
Carcinoma <i>in situ</i>	E	20	Vulva	+	–	–
	F	40	Vulva	+	–	*
	G	27	Vulva	–	–	–
	H	44	Vulva	–	–	–
	I	42	Vulva	–	ND	–
	J	77	Vulva	+	ND	+
	K	32	Vulva	+	–	–
	L	84	Vulva	–	ND	–

+, HPV sequences detected; –, HPV sequences not detected; ND, not determined.

* When the small amount of viral DNA seen in stringent conditions (Fig. 2, patient F) is compared with the large amount seen in less stringent conditions (Fig. 1, patient F), there are two possible explanations: either there is more than one type of HPV present in this tumour and HPV-6 represents a very low percentage of the total viral DNA or there is such a high concentration of viral DNA in the sample that low level cross-hybridization has occurred. As neither possibility can be conclusively excluded by the data presented, we prefer not to classify the HPV type present.

role for papillomaviruses. Since several patients had a previous history of wart disease it is possible that latent virus or viral DNA remained in the skin of these patients after eradication of the wart disease. In support of this contention is the identification of HPV DNA in normal skin of some patients with EV disease (R.S.O. *et al.*, in preparation). On the other hand, the history of previous wart disease was not a criterion for choosing the patients. Our original interest was in patients with multifocal tumours which clinically resembled warts. These studies, therefore, also include patients that exhibited no previous history of wart disease, and we similarly found HPV DNA associated with their tumours. It is conceivable that either these patients may have had small lesions that were undetected or they were infected subclinically. Nevertheless, our studies indicate that the entire spectrum of anogenital tumours, from

benign to malignant, can carry free HPV DNA genomes. More patients must be studied to understand the prevalence of this association. Restriction endonuclease mapping and other molecular studies must be performed to characterize the virus type. As many patients with anogenital wart disease do not develop genital malignancies, other factors may be involved. Host immunity, co-carcinogens and infection with other virus groups may also have a pathogenic role with HPV in the process of malignant conversion.

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Note added in proof: After submission of this manuscript, Green *et al.* reported the presence of HPV-specific DNA in several different anogenital carcinomas⁸.

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Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11

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The development of DNA-mediated gene transfer techniques^{1,2} has made it possible to identify transforming genes present in certain human tumour cells. Such genes have been shown to induce morphological transformation when used to transfect suitable assay cells^{3–10}. Recently a transforming gene has been isolated by molecular cloning techniques from the T24 (ref. 11) and EJ (ref. 12) human bladder carcinoma cell lines^{10,13,14}. This bladder carcinoma oncogene has been shown to be of human origin, less than six kilobase pairs (kbp) in size, and closely related to the *onc* genes (*v-bas* and *v-ras*) of BALB and Harvey murine sarcoma viruses^{15–17}. These transforming retroviruses arose in nature by transduction of cellular genes from mouse and rat cells, respectively¹⁸. To understand better the relationship of the T24 oncogene with other human cellular genes, we have determined the chromosomal location of its normal allele within the human genome. We show here that it is carried on chromosome 11 in normal cells.

Somatic cell hybrids were constructed between various human cell lines and either mouse or Chinese hamster cells. Such hybrid cells segregate human chromosomes and will only show the presence of the corresponding human gene when the relevant chromosome has been retained. Thus, comparison of the presence or absence of the human gene with the known karyotype of a number of somatic cell hybrids allows assignment of the gene to a particular human chromosome.

To map the normal allele (*c-bas*) of the T24 oncogene, a 3.0-kbp *SacI* subfragment containing *v-bas* related sequences¹⁷ was used as a probe. As shown in Fig. 1, this probe detected a 20-kbp fragment in *EcoRI*-cleaved human placenta DNA. Using stringent hybridization conditions, hybridization with related genes present in either mouse or Chinese hamster DNA was not found. The 20-kbp fragment was observed in 4 of 18 somatic cell hybrids (Fig. 1) in one series (Fig. 2) examined. Analysis of two additional series of hybrid cell lines (*b* and *d*, Table 1) showed that hybridization to the T24 probe correlated

only with the presence of human chromosome 11.

To confirm assignment of the normal allele of the T24 oncogene to chromosome 11, six hybrid cell lines were subcloned, including three lines which contained chromosome 11. This procedure allowed further human chromosome segregation. Spontaneous loss of chromosome 11 was observed in many subclones (14/32) of the three parental lines originally retaining this chromosome. However, detection of T24 oncogene sequences by molecular hybridization in each series of subclones correlated with the presence of chromosome 11 (Table 1c) and therefore the normal human homologue of the T24 oncogene can be unambiguously assigned to this chromosome.

Previously assigned markers carried on human chromosome 11 include lactate dehydrogenase A (LDH-A) and esterase-A4 (EsA4) which have been regionally localized to the short arm (11p1203–11p1208) and proximal portion of the long arm (11cen–11q22) respectively¹⁹. The presence of the normal allele of the T24 oncogene correlated with expression of human LDH-A in all hybrids. However, human EsA4 was detected in four additional hybrid lines (Table 1b) which did not contain this oncogene or human LDH-A. The chromosome 11 break point in these four lines has not been determined by karyotypic analysis, but a fragile site has been previously described on the proximal portion of the long arm (11q13) of chromosome 11 in cultured cells²⁰. Our results currently exclude the distal portion of the chromosome 11 long arm as the locus of the T24 transforming gene.

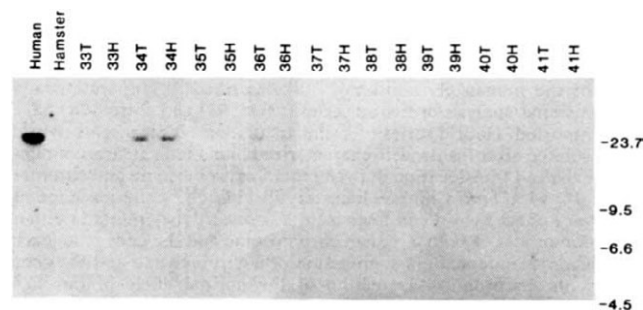


Fig. 1 Hybridization of the normal allele of the T24 oncogene to *EcoRI*-digested DNA from human–Chinese hamster somatic cell hybrids. Human and Chinese hamster control DNAs were run in lanes 1 and 2 respectively. Lanes 3–20 contained DNAs from 18 somatic cell hybrids of series *a* (Table 1). After size-fractionation by electrophoresis, DNAs were transferred to nitrocellulose filters and hybridized to a nick-translated 3-kbp cloned insert derived from the normal allele of the T24 oncogene¹⁷. The positions of *HindIII*-digested λ DNA fragments are indicated on the right.

Normal cellular alleles of the T24 oncogene can be transforming genes in a variety of conditions. The transduction of mouse or rat cell homologues of this gene by retroviruses had led to the creation of BALB- and Harvey-murine sarcoma viruses. Recent studies have shown that the normal human gene itself can be manipulated *in vitro* by the addition of retroviral long terminal repeat sequences so as to acquire transforming activity²¹. Only minor genetic alterations lead to activation of this gene as a transforming gene in T24 bladder carcinoma cells. By restriction mapping and heteroduplex analysis, the normal gene could not be distinguished from the T24 oncogene in the region required for transforming activity¹⁷, excluding any major insertion, deletion or major rearrangement of sequences. In fact, recent findings indicate that a point mutation affecting the twelfth amino acid residue from the amino terminus of the T24 oncogene product accounts for its transforming activity^{22,23}.

The potential importance of *onc* genes of transforming retroviruses for understanding basic mechanisms involved in malignant transformation, has recently led to the chromosomal mapping of several of these genes. Thus, the human homologue of *v-sis*, the *onc* gene of simian sarcoma virus, has been assigned to chromosome 22 (refs 24, 25) while *mos* (ref. 26), *fes* (refs 27, 28), *myb* (ref. 27), and *abl* (ref. 28) have been mapped to chromosome 8, 15, 6 and 9, respectively. Our chromosomal assignment of the normal allele of the T24 oncogene to chromosome 11 represents the first mapping of a cellular gene shown to be activated as a transforming gene in a human tumour. Knowledge of the locations of this and other *onc*-related genes has obvious importance for determining the relationship, if any, between known chromosomal aberrations in naturally occurring tumours and oncogenes found on such chromosomes.

Table 1 Segregation of the normal allele of the T24 oncogene with specific human chromosomes

Series	Total hybrids	T-24 allele positive	% Discordancy	
			Chromosome 11	Other chromosomes
a	18	4	0	≥11
b	19	3	0	≥16
c	56	18	0	≥16
d	22	10	0	≥23

Assignment of the normal allele of the T24 oncogene to human chromosome 11 is based on analysis of the segregation of this gene with specific human chromosomes in human-rodent somatic cell hybrids. Cell pellets were simultaneously prepared for DNA isolation and isoenzyme analyses from three series of somatic cell hybrids (a, b, d) and subclones of six hybrid cell lines (c). The 3.0-kbp *SacI* probe was hybridized to nitrocellulose filters containing *EcoRI*-digested, size-fractionated cellular DNAs. The human chromosomes present in each hybrid cell line were determined from starch gel electrophoretic analyses^{31,32} of isoenzyme markers that have been previously assigned to each of the human chromosomes¹⁹ as described²⁴. Preparation and chromosomal analysis of hybrid series a (ref. 33) and d (ref. 24) have been reported. Hybrid series b is similar to a, and it represents hybrid lines isolated after fusing well-characterized human fibroblasts containing a balanced translocation between the X chromosome and chromosome 14 with *hprt*⁻Chinese hamster fibroblast³³. c Series denotes subclones of six hybrid cell lines from d. Concordancy refers to either retention or loss of both a human chromosome and the gene from each line, whereas independent segregation of a chromosome and the gene represents discordancy. Detection of the normal allele of the T24 oncogene could be correlated with the presence or absence of human chromosome 11 in all hybrid cell lines. The minimum discordancy between segregation of all other human chromosomes and the oncogene is shown with the following exceptions: a, 0% and 6% discordancy with chromosomes 13 and 16, respectively; b, 0% and 11% discordancies with chromosomes 3 and 4 and chromosome 10, respectively; c, 5, 7, 9 and 9% discordancies with chromosomes 22, 1, 6 and 9, respectively. The long arm of chromosome 11 alone (EsA4 marker) did not segregate concordantly with the T24 oncogene in series b (see text).

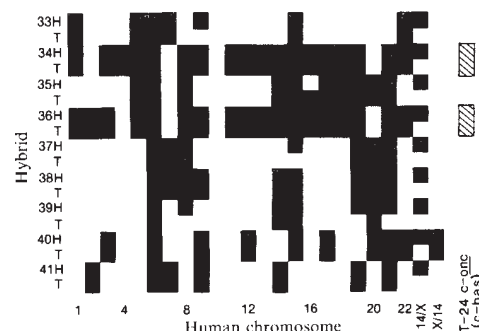


Fig. 2 Distribution of specific human chromosomes in human-hamster cell lines of a (Table 1). Hybrids were isolated after fusing well characterized human fibroblasts (GM0073) containing a reciprocal X;14 translocation with *hprt*⁻ Chinese hamster fibroblasts and cloning each independent line twice³³. Individual hybrid cell lines are represented on the ordinate and H or T indicates expansion of that cell line in HAT (100 μ M hypoxanthine, 1 μ M aminopterin and 16 μ M thymidine) or 50 μ M 6-thioguanine, respectively. Specific human chromosomes are represented on the abscissa. Solid boxes indicate the presence of a particular human chromosome in a hybrid line, and open boxes indicate absence of the chromosome. The presence of the normal allele of the T24 oncogene is shown by hatched boxes in the last column.

The relevance of human chromosome 11 to cancer-associated genes has previously been restricted to the association of a deletion on this chromosome with Wilms' tumour, a malignant renal tumour of children²⁹. The deletion has been localized to 11p13 by binding techniques³⁰. This deletion could lead to loss of some normal function or to a rearrangement that might result in gene activation. Although we cannot yet precisely localize the T24 oncogene to this specific region, it is tempting to speculate that an association may be involved since the oncogene segregates concordantly with a short arm (11p) marker and discordantly with a long arm (11q) marker for this chromosome.

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Low Ca^{2+} impedes cross-bridge detachment in chemically skinned *Taenia coli*

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Muscle force is generated by cycling cross-bridges between actin and myosin filaments. In smooth muscle, cyclic attachment and detachment of cross-bridges is thought to be induced by a Ca^{2+} - and calmodulin-dependent myosin light chain kinase which phosphorylates myosin. The relaxation that occurs after Ca^{2+} removal is usually ascribed to dephosphorylation of myosin by a phosphatase as non-phosphorylated myosin is unable to form force-generating cross-bridges¹. Recently, Dillon *et al.*² claimed, however, that dephosphorylation of attached cross-bridges may impede cross-bridge detachment, thus forming so-called 'latch bridges'. Here we present evidence that after a Ca^{2+} - and calmodulin-induced contraction of chemically skinned guinea pig *Taenia coli*³, the rapid removal of Ca^{2+} impedes the detachment of the myosin cross-bridges from the actin filament; force can then be maintained without energy consumption. The extremely slowly detaching cross-bridges which maintain the force after Ca^{2+} removal may indeed correspond to the 'latch bridges' mentioned above.

The Ca^{2+} -jump technique⁴ allows the Ca^{2+} concentration to be changed in small bundles of chemically skinned muscle fibres within fractions of a second. Compared with this time scale, the mechanical response in chemically skinned *T. coli* is much slower; the broken line marked $\pm \text{Ca}^{2+}$ in Fig. 1 shows the time at which the Ca^{2+} concentration was changed from $<10^{-8}$ M to 10^{-5} M and vice versa. The force generated by the fibre (solid line) shows a marked delay in response to Ca^{2+} . It is conceivable that this delayed relaxation was due to an internal viscoelasticity independent of the actin-myosin interaction. Such a 'passive' viscosity would probably not be affected by inorganic phosphate. However, the relaxation was markedly accelerated after addition of 6 mM inorganic phosphate to the incubation solution.

Figure 1 shows that in contrast to the force (solid line), the ATPase activity (open circles) decreased immediately after Ca^{2+} removal. Thus, the high force maintained after Ca^{2+} removal must be ascribed to non-cycling but attached and force-maintaining cross-bridges. The simplest interpretation of our findings is that the rate constant of the cross-bridge detachment is similar to the rate constant of tension decrease, that is, $\sim 0.1 \text{ min}^{-1}$. From the ATPase activity (Fig. 1) and the total myosin concentration in this type of muscle⁵, the turnover rate for the actin-myosin interaction was estimated to be $\sim 0.25 \text{ s}^{-1}$ at 10^{-5} M Ca^{2+} , assuming that all myosin molecules are interacting with actin and that they interact with only one head at the same time. As ATP splitting requires a complete cross-bridge cycle (including detachment and reattachment of the cross-bridge), the turnover time of the cross-bridge cycle must be longer than the time needed for cross-bridge detachment. Thus the detachment rate of the cross-bridges after Ca^{2+} removal (estimated

Fig. 1 Force and ATPase activity of chemically skinned *T. coli* from guinea pig. The solid line shows the generated force, the open circles the simultaneously measured ATPase activity ($\mu\text{mol ADP l}^{-1}$, fibre vol s^{-1}). The dashed line marks the time at which the Ca^{2+} concentration was changed from $<10^{-8}$ M to 10^{-5} M ($+\text{Ca}^{2+}$) and vice versa ($-\text{Ca}^{2+}$). The time at which 6 mM phosphate was added to the incubation solution is indicated by the broken line marked P_i . The 'tension cost' has a maximum at the start of the contraction (see ref. 9). ATPase activity was measured using an NADH-coupled optical assay method; in this assay the consumption of NADH corresponds to ADP production. A fibre bundle (0.3 mm diameter, length 7 mm) was mounted in a narrow chamber (diameter 0.8 mm) which was perfused with an ATP-salt solution ($\text{pH } 6.7$, $T = 20^\circ\text{C}$) consisting of: 30 mM imidazole, 7.5 mM ATP, 10 mM MgCl_2 , 1 mM NaN_3 , 1 mM phosphoenolpyruvate, 4 mM EGTA, CaEGTA, 100 U ml^{-1} pyruvate kinase, 140 U ml^{-1} lactate dehydrogenase, 0.6 mM NADH, 0.2 mM $\text{P}^{1,5}$ -di(adenosine-5')-pentaphosphate, 2 mM dithioerythritol, 0.1 μM calmodulin (for the effect of calmodulin see ref. 3). To measure the NADH consumption, the perfusion was stopped for 30 s (see inset: upper trace, NADH concentration in the chamber; lower trace, force. The time at which the Ca^{2+} concentration was changed from 10^{-5} M to $<10^{-8}$ M is marked by an arrow). The change in the NADH fluorescence intensity with time in both chamber and fibre was recorded with a Zeiss microscope photometer. After the measurement, the chamber was perfused again with fresh solution, and the procedure was repeated.

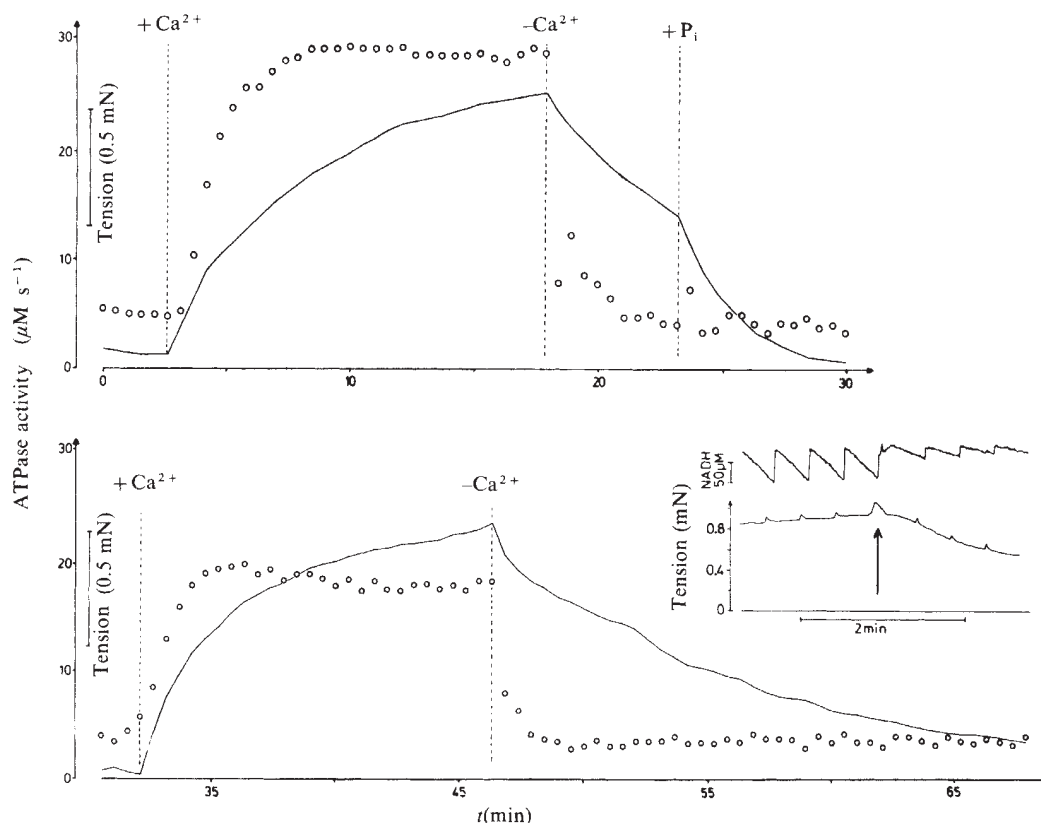
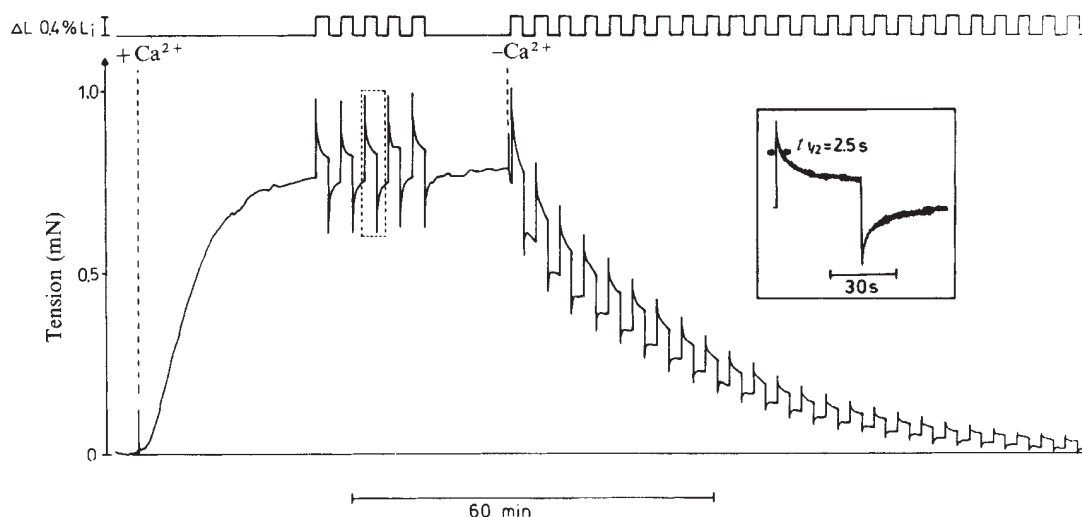


Fig. 2 Force response of a repetitively stretched and released skinned *T. coli*. The upper trace shows the length signal, the lower trace the corresponding force. The broken line marks the time at which the Ca^{2+} concentration was changed from $<10^{-8}$ M to 10^{-5} M ($+\text{Ca}^{2+}$) and vice versa ($-\text{Ca}^{2+}$). The inset shows, with an expanded time scale, the force transient of one stretch-release cycle. Conditions as described in Fig. 1 legend. ΔL , 0.4% L_i = length change as % of the initial length (L_i).



above to be $\sim 0.1 \text{ min}^{-1}$) must be over 100-fold slower than the detachment in the presence of Ca^{2+} . It has been concluded from biochemical studies, however, that ATP may also be split by myosin-S1 being attached to actin⁶. Thus the cross-bridge cycle of attachment and detachment could be much slower than indicated by the rate of the ATP splitting process. To obtain a different estimate for the cross-bridge detachment rate, contracted fibre bundles were stretched and released repetitively (see Fig. 2: upper trace, muscle length; lower trace, the corresponding force). A plausible interpretation of the force transients obtained is that after the length change, the force changes (with a half time ($t_{1/2}$) of ~ 2.5 s) until the cross-bridges that are attached at the moment of the length change are replaced by others. The kinetics of this process is the same for small stretches and for small releases, indicating that it also reflects the cross-bridge kinetics of the isometric contracting state. If this is so, and if the detachment limits the rate of the force recovery after the length change, then the rate of cross-bridge detachment in the Ca^{2+} -activated state presumably must be greater than $\sim 0.5 \text{ s}^{-1}$ (compared with 0.1 min^{-1} after Ca^{2+} removal). The tension recovery following length perturbations after Ca^{2+} removal seems to be slower and less complete (see also Schneider *et al.*⁷). This finding is also consistent with a slow detachment of cross-bridges after Ca^{2+} removal.

The results reported here indicate that after Ca^{2+} removal, cross-bridge detachment occurs much more slowly than in the presence of Ca^{2+} . The absence of Ca^{2+} obviously inhibits not only the myosin cross-bridge attachment to actin but it also impedes cross-bridge detachment from actin (see also 'catch' state in the Byssus retractor⁸). Whether this is a direct effect of the lack of Ca^{2+} or an indirect effect due to dephosphorylation of myosin remains to be determined. In any case, after Ca^{2+} removal, non-cycling cross-bridges or those that are still attached cause the high force to be maintained with very low ATPase activity.

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Orientation of spin labels attached to cross-bridges in contracting muscle fibres

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Electron micrographs showing different cross-bridge orientations in different states of muscle fibres, and X-ray diffraction patterns indicating axial cross-bridge disorder in contracting muscle first suggested that force generation in the contracting muscle involved a change in orientation of the myosin heads that form cross-bridges between thick and thin filaments^{1,2}. This has been supported by subsequent work; the myosin molecule has the required flexibility for changes in orientation^{3,4}. The orientation of muscle tryptophans and of probes attached to the myosin heads of permeable muscle fibres depends on the state of the muscle⁵⁻⁹. Recently, fluorescence polarization fluctuations and time-resolved X-ray diffraction patterns have suggested that cross-bridges of a contracting muscle can rotate^{10,11}. We have used electron paramagnetic resonance (EPR) spectroscopy to monitor the orientation of spin labels attached specifically to a reactive sulphhydryl on the myosin heads in glycerinated rabbit psoas skeletal muscle. Previously, it has been shown that the paramagnetic probes are highly ordered in rigor muscle, with a nearly random angular distribution in relaxed muscle¹². We show here that during the generation of isometric tension, $\sim 80\%$ of the probes display a random angular distribution as in relaxed muscle while the remaining 20% are highly oriented at the same angle as found in rigor muscle. These findings indicate that a domain of the myosin head does not change orientation during the power stroke of the contractile interaction.

The maleimide spin label (MSL) can be reacted selectively with a reactive sulphhydryl on the myosin head (SH_1) in a glycerinated rabbit psoas muscle fibre^{12,13}. The EPR spectra can be used to determine directly and unambiguously the angular distribution of the probes relative to the magnetic field^{12,13}. Here we have used spin labels attached to SH_1 to

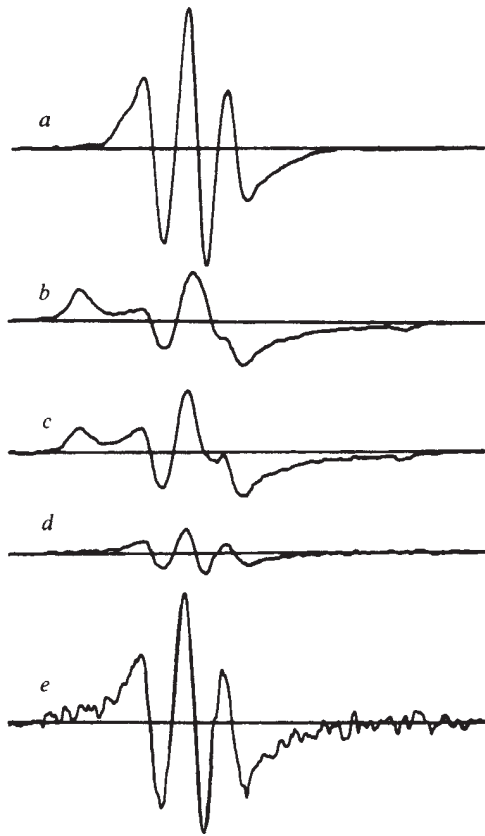


Fig. 1 EPR spectra of spin labels attached to the myosin heads during rigor (*a*), relaxation (*b*) and isometric tension (*c*). Spectrum *d* is a difference spectrum, obtained by subtracting 81% of spectrum *b* from spectrum *c*. This difference spectrum is shown again in *e*, amplified by 5.3 so that it represents the same number of probes as spectra *a*–*c*. Glycerinated rabbit psoas fibres were labelled with MSL by the procedure of Thomas and Cooke¹² except that the label concentration was increased to 0.5 mM, the pH was 6.5, and the duration of labelling was 10 min. Approximately 50–70% of the heads were reacted at the SH₁ as judged by extraction of myosin and measurement of ATPase activity in the presence of EDTA. Spectra were obtained at 24 °C with a Varian E109E spectrometer interfaced to a Northstar computer. The E238 cavity was modified to accept a capillary (1.2 mm inside diameter) parallel to the applied magnetic field. About 100 fibres, in bundles of 5–10, were secured on either end with surgical thread and pulled into the capillary, almost filling it. Solution was allowed to flow past the fibres at 0.3 ml min⁻¹ (that is, with a velocity of 2–5 cm s⁻¹), aiding perfusion of the bundles. No differences in the spectra were seen when the bundle size or flow rate was increased or decreased by a factor of 2. Fibres in rigor were bathed in 'rigor buffer' (0.12 M KCl, 5 mM MgCl₂, 2 mM EGTA and 20 mM MOPS, pH 7.0). Relaxed spectra were obtained by addition of 5 mM ATP, 20 mM CP and 0.4 mg ml⁻¹ creatine kinase to the rigor buffer. Contraction was induced by adding 2 mM CaCl₂. After contraction the fibres were returned to rigor solution, and data were used only when the reappearance of a well ordered spectrum indicated that sarcomere heterogeneity had not developed during contraction. The baseline for each spectrum is 100 G wide.

investigate cross-bridge orientation during isometric contraction of skeletal muscle.

Figure 1 shows the EPR spectra of MSL-labelled muscle fibres held isometrically in conditions of rigor (Fig. 1*a*), relaxation (*b*) and tension (*c*). The spectrum of fibres in rigor indicates that the probes are highly oriented, with a gaussian angular distribution of 15° (full width at half maximum) centred about an angle of 82 ± 1° with respect to the fibre axis¹². When ATP is added to relax the fibres, the EPR spectrum shows that the

angular distribution of the probes is almost isotropic¹² (Fig. 1*b*). Addition of Ca²⁺ induces tension (see Fig. 1*c*); this spectrum can be shown to be a superposition of the spectra obtained in rigor and relaxation. When 81% of the spectrum in relaxation (Fig. 1*b*) is subtracted from that in tension (Fig. 1*c*), the difference spectrum is identical to that of rigor fibres (Fig. 1*d*, *e*). Subtraction of different fractions (not shown) shows clearly that the correct disordered fraction lies between 75 and 85%. Thus, under isometric tension, 80 ± 5% of the myosin heads have probes disoriented as in relaxed muscle, while the probes on the remaining heads are highly oriented similar to fibres in rigor. The most dramatic aspect of Fig. 1*c* is that no new probe angles, different from those seen in rigor or relaxation, are observed. The resolution of the spectrum is high. If over 5% of the probes assumed a new, well defined angle differing from the rigor angle by >10°, or if over 10% of the probes were less disordered than in relaxation (that is, distributed over <90°) they would have been detected.

A first question which arises from the spectrum of Fig. 1 is whether the 'rigor-like' component of the spectrum obtained during force generation represents a state in the normal cycle of the cross-bridge or whether it is due to some portion of the sample that is in rigor because of an inadequate supply of ATP. We have addressed this question by varying the concentration of ATP or creatine phosphate (CP), and by observing the speed at which small molecules can diffuse into the fibres. Figure 2 shows the fraction of disoriented probes as a function of the ATP concentration. The fraction of disoriented probes approaches 75% as the concentration of the substrate approaches infinity. A similar result was obtained when the concentration of CP was varied. Thus, the myosin heads seem to be saturated with substrate in the conditions used in Fig. 1. The conclusion was further strengthened by several additional experiments. Following a change in the perfusing solution from a rigor solution to a relaxing solution, the spectral changes occurred exponentially with a time constant of 5 s, showing a rapid rate of substrate diffusion into the fibres. If the flow of the perfusing solution was abruptly stopped during contraction, no increase in the rigor component was observed for over 40 s, indicating that the fibres contain sufficient substrate for this

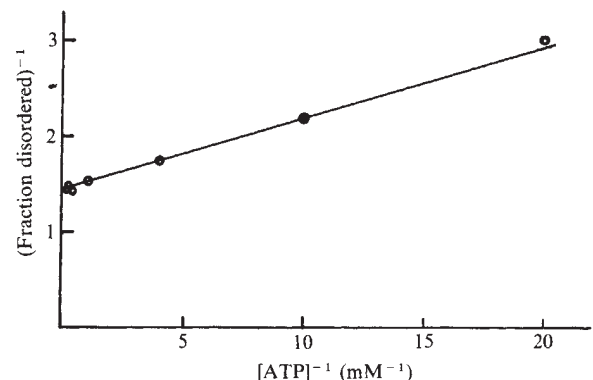


Fig. 2 The fraction of disoriented probes as a function of the ATP concentration over the range 50 µM to 10 mM; conditions are the same as in Fig. 1*c*, except for ATP concentration conditions. The fractions of disoriented and oriented probes are proportional to the heights of the first (P₁) and second (P₂) spectral peaks, respectively. Because a given peak height for P₁ represents twice as many probes as P₂ (see Fig. 1*a*, *b*), the fraction of disoriented probes is 2P₁/(2P₁ + P₂). The inverse of this fraction is plotted in Fig. 2, as a function of the inverse of the ATP concentration. If the fraction of oriented probes decreased to zero as substrate increased the data should extrapolate at the ordinate to 1, the value for relaxed fibres. This does not occur, and the extrapolated value shows that ~25% of the probes remain oriented as the substrate concentration approaches infinity.

time period and that the magnitude of the rigor-like component is not due to a balance between the rate of substrate diffusion into the fibres and its consumption.

We conclude that all the myosin heads in our fibres were in the presence of saturating ATP, therefore that the different spectral components corresponded to different states in the force-producing cross-bridge cycle. Thus, in isometric tension, the probed domain in each myosin head spends about 80% of its time in a state with disorientation identical to that of relaxation, and about 20% of its time in a state having an orientation identical to that of rigor. Reaction with the label decreased the active tension of the fibres by ~20%. Further reaction of the fibres with the non-paramagnetic *N*-ethylmaleimide (2 mM) for 2 h eliminated both active tension and the oriented component of the active fibres. Thus, the oriented component seems to represent active cross-bridges, and its magnitude may be decreased by 20% due to the inactivation of some cross-bridges during labelling.

Although the EPR spectra in Fig. 1 report most directly on orientation, they provide information about the fraction of myosin heads attached to actin. Other work, using conventional EPR to measure orientation¹² (as described here), and saturation transfer EPR to measure motion¹³⁻¹⁵, have shown that probes are highly oriented and immobile when myosin is attached to actin (in fibres or myofibrils in rigor, or in actomyosin) but are highly disoriented and mobile (on the μ s scale) when myosin is detached from actin (in fibres at long sarcomere lengths or in relaxation, or in purified myosin filaments). Saturation transfer EPR experiments on myofibrils show that most of the probes are as mobile during contraction as in relaxation, suggesting that most are detached¹³. Thus, the most straightforward interpretation of the data in Fig. 1 is that during tension generation, the myosin head spends ~20% of its time attached to actin, and that for most of that time the orientation of the probed region is identical to that in rigor. In principal, we cannot exclude the possibility that some of the disordered probes are attached in either relaxation or tension, but this seems unlikely in view of the previous observations that they have the same degree of disorder and motion as in fibres stretched beyond overlap. The fraction of attached heads in contracting fibres has been estimated using various techniques. Fibre stiffness and X-ray diffraction have suggested, respectively, that roughly 70% and 50% of the heads are attached^{16,17}. However, values close to that found here are obtained from

calculations based on the ATPase activity rate of isotonically contracting frog fibres (ref. 18 and W. Bialek and R.C., unpublished), and from a study of the motion of a myosin-bound spin label¹⁹.

Information on cross-bridge dynamics has been obtained from measurements of the transients in tension or sarcomere length that follow step changes in muscle length^{20,21}. Although there are many possible molecular explanations of these and other transient phenomena, they strongly suggest that the predominant attached states during isometric tension occur early in the power stroke, not at the end^{20,23}. Nevertheless, our EPR spectra indicate that the predominant probe orientation is identical with that of rigor, the state which is usually assumed to be at the end of the power stroke. These results suggest, therefore, that states early and late in the power stroke have the same probe orientation, that is, the probed region of myosin does not rotate during the power stroke. This is supported by the observation that stress does not alter the orientation of spin labels on myosin heads in rigor fibres, a result which shows that the angle of the spin label is not linked to the force supported by the fibre²⁴. It is possible that the probe is oriented relative to the myosin head at such an angle that myosin rotation does not affect the probe orientation, but this is extremely unlikely in light of the high resolution of the rigor-like component of the contracting fibres; neither the centre of the distribution nor its width differs from that of rigor by more than 1°.

There are many different models that fit the conclusion of this study. One possibility is that the entire myosin head maintains a constant orientation during the power stroke, while some other part of myosin shortens; one example of such a model has been proposed²⁵. Alternatively, the probed region of myosin may remain rigidly oriented on actin, while another domain of the myosin head does rotate. There is, as yet, insufficient evidence to exclude either possibility.

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BOOK REVIEWS

Mapping the evolution of biochemistry

Frederic L. Holmes

IN THIS first full-length study of the institutional structures within which the family of sciences known today as "biochemistry" have grown to maturity, Robert Kohler describes the formation, development, and sometimes the decline, of the various administrative organizations within which that field has been practised and taught. Kohler bases his account on an impressive array of unpublished institutional and individual archives; his aim is to reconstruct representative events underlying what he defines as "the changing political map of scientific disciplines".

Beginning with the origins of physiological chemistry in Germany between 1840 and 1900, Kohler recounts how, after a promising start, that field failed to develop independent institutional foundations. The subject was pursued instead within other disciplines such as physiology and pathology. Even though German scientists exerted much of the leadership in biochemical research then and on into the early twentieth century, they failed, according to Kohler, to build a discipline. Equally, in Great Britain physiologists resisted the formation of separate departments of physiological chemistry, even though they regularly hired organic chemists to fill the posts in physiological chemistry. The major exception to this pattern was the Cambridge school of general biochemistry, which Frederick Gowland Hopkins was able to establish in the 1920s with the aid of an outside foundation.

Until the 1930s, Germany and Britain provided most of the leading scientists in biochemistry. The United States remained, with some notable exceptions, a research backwater. Nevertheless Kohler devotes over two-thirds of his book to American biochemistry between 1900 and 1940. His justification for this apparent imbalance is that in the United States biochemists were able to achieve the independence which eluded their counterparts in Europe. Kohler shows in detail how this feat was managed in representative American schools. It was the reform movement in American medical schools at the turn of the century, especially the drive to introduce "scientific" medicine, that provided physiological chemists with their opportunities; and it was, Kohler repeatedly emphasizes, because the biochemists established a "service role" teaching medical students that they were able to command

From Medical Chemistry to Biochemistry: The Making of a Biomedical Discipline. By Robert E. Kohler. Pp.399. ISBN 0-521-24312-2. (Cambridge University Press: 1982.) £22.50, \$34.50.

the political and financial backing necessary to maintain departments, to support teaching and research positions, and to find jobs for those they trained. At the same time, most efforts to introduce biochemistry as a broadly-based, biological subject into scientific schools and undergraduate colleges failed for lack of a stable service role.

Kohler skilfully uses selective case-histories of prominent medical schools to build up a picture of what he regards as the typical patterns of institutional development. Particular departments which departed from these patterns he treats as "deviant" cases, even though the groups which worked in them included some of the most distinguished and influential leaders in the field. He presents his case-histories in short, well-paced narratives, including lively character sketches of the personalities involved, with entertaining descriptions of the manoeuvres in which they often engaged as "discipline builders" and of the disasters which overcame some of the less capable entrepreneurs.

At his best Kohler writes with a felicitous style; sometimes, however, he resorts to distractingly colloquial language and to deliberately political metaphors, such as "physiologists developed an expansive imperialistic program", and "British physiologists were determined opponents of home rule for physiological chemistry". These phrases are designed to reveal scientists as politically motivated and fundamentally no different from any other self-interest group. Use of such language, however, is a cheap way to make the point. It does so merely by colouring the historical figures with the historian's conception of them, without taking the trouble to demonstrate that these people were not primarily motivated by a commitment to their scientific calling.

Kohler's account, based as it is on extensive archival sources, is convincing. There is reason to be wary, however, about the reliability of some of the factual detail. To take one example, chosen because it is familiar to me, Kohler refers briefly to aspects of the career of Hans Krebs in seven different places in his book. Three of these

passages contain mistakes. Kohler says (p.71) that Krebs was given a research position at Sheffield in 1938, whereas the correct date was 1935. He writes (p.326) that Krebs made "a smooth transition from pathology to biochemistry in the mid-1930s"; in fact, Krebs was never a pathologist, and he had been involved in biochemical research since 1926. Finally, in a footnote on p.386, Krebs is described as "one of the first outside Beadle's circle to do biochemical genetics", despite the fact that Krebs was never personally involved in research in that field. These are not trivial errors, for in each case the author is illustrating a general point which depends upon the misinformation in question.

The aim of *From Medicinal Chemistry to Biochemistry* is far more ambitious than simply to map out the institutional setting within which the discipline of biochemistry developed, for Kohler's thesis is

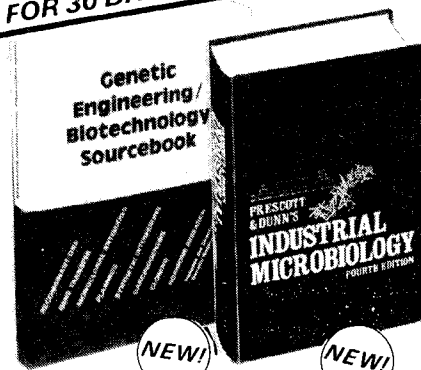
that biochemists' programmatic conceptions of their discipline were shaped by institutional contexts and relationships, such as channels of recruitment, political alliances, and service roles.

Repeatedly he views "research programmes" as arising from local circumstances, tied to strategies appropriate to success within a particular medical school, chemistry department or other institutional arrangement.

It is certainly true that such local conditions influence the type of science practised within individual organizations, but only in the sense that they define which of the possible niches within the broader research stream a particular local group can attempt to occupy. It is not the local patrons who adjudicate whether the research carried out within their organizations is significant enough to merit recognition; rather it is colleagues pursuing similar problems in other centres — comprising the international community, or "invisible college" of a particular speciality — who make such judgements. We cannot evaluate the degree to which institutions shape research programmes until we have a thorough grasp of what is being shaped. Kohler touches frequently upon the research areas that the biochemists in his story pursued, but he never penetrates deeply enough into these topics to assess the interplay of influences which directed them.

In Joseph Fruton's masterly *Molecules and Life* (Wiley, 1972) and Marcel

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Florkin's more encyclopaedic *A History of Biochemistry* (in *Comprehensive Biochemistry*, Vols 30-33b, Elsevier; 1972-1982) we have two very useful descriptions of the main lines of investigation that have characterized the biochemical family of sciences over the past two centuries. Kohler's work on institutions complements these accounts, but he chooses to dismiss the work of Florkin and Fruton as representative of the allegedly outmoded approach of an earlier generation of "insiders". Without their help, however, and much more besides in the way of detailed studies of specific areas of biochemistry, Kohler's assertions about the shaping force of institutions will remain empty generalizations.

By reconstructing the institutional contexts within which biochemistry developed, Kohler has made progress towards an eventual description of the contours of that subject. A modern scientific discipline is, however, an enormously complex cluster of individual leaders and followers, schools, sub-specialities, literature, methods, concepts, traditions, careers, solved and unsolved problems, evolutionary and revolutionary events, as well as a set of institutional organizations. To understand the interplay of all these factors is a formidable task, the magnitude of which social historians of science have scarcely begun to appreciate. □

Frederic L. Holmes is Chairman of the Section of History of Medicine, Yale University School of Medicine, and President of the History of Science Society.

Programs for seeing

Oliver Braddick

Computer Vision. By Dana H. Ballard and Christopher M. Brown. Pp.523. ISBN 0-13-165316-4. (Prentice-Hall: 1982.) £33.95, \$39.95.

BALLARD and Brown intend their book "... both as a senior/graduate level text and as a useful reference to those building vision systems". These are admirable and ambitious goals in a field where most books so far have been symposium proceedings or collections of papers, and where the primary literature tends to be a bibliographic jungle of technical reports and conference contributions.

The authors have certainly succeeded in providing a valuable work of reference. The book contains a compendium of the techniques that have been found useful at every level of computer vision systems. If you need an outline of the different possible tessellations of the image plane, the representation of illuminated surfaces in gradient space, chain codes for boundary

representation or graph-matching algorithms, these and much more are presented at a sufficiently basic level that they will make the research literature much more accessible to the student or outsider. The frequent use of concrete examples from the programming of vision problems helps to overcome the risk of daunting abstractions and to make clear that the authors' accounts of some general artificial-intelligence issues, such as production systems and semantic nets, do have their relevance to the specific needs of vision.

The goal of making computer programs that obtain knowledge of the world from optical information can be approached from either end. One approach is the search for operations which are effective in extracting from the optical input that information which purely and reliably conveys the significant properties of the world, stripped of the incidental variations of viewpoint, illumination, noisy imaging processes and so on. The other is to realize the richness of prior knowledge that human beings bring to perceptual tasks, and to seek ways in which programs can efficiently use such knowledge to organize the noisy deluge of optical information.

Both approaches are fairly represented in Ballard and Brown's book. Someone planning a vision system might reasonably ask how far they can expect to take "bottom-up" information extracting procedures, and what kinds of otherwise intractable ambiguities or overloads may be dissolved by the "top-down" application of world knowledge. Certainly this is the sort of issue which those interested in human vision hope will be illuminated by computer vision studies. However, the book is less strong on providing this kind of overview. "Top-down" versus "bottom-up" processing may be too over-arching an issue for much resolution of it to be expected, but similar criticisms could be made on a smaller scale. A wealth of techniques is offered, but rather less evaluation of how well they handle various kinds of problem, or consideration of *why* a particular method should be relatively effective or ineffective. The latter is an ambitious demand; but perhaps a field which is still looking for the necessary organizing principles cannot yet sustain a true textbook.

These problems may be a reflection of a more general tension within computer science: is it the body of guidelines to effective practice which underlies a technology, or the body of results about the nature of the world which constitutes a science? Many people are convinced that there is, in the making, a science of how both artificial and biological vision systems can work: Ballard and Brown provide a rare guidebook to trails on the slopes of this unclimbed peak. □

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Yet more population modelling

Robert H. Smith

Modelling Fluctuating Populations. By R.M. Nisbet and W.S.C. Gurney. Pp.379. ISBN 0-471-28058-5. (Wiley: 1982.) £19.50, \$47.50.

THE APPEARANCE of yet another book on population modelling written by yet more mathematicians/physicists/engineers with an enthusiasm for ecology is, on a probabilistic basis, unlikely to arouse much interest amongst ecologists. With a few notable exceptions, such texts are mostly regarded as excuses for mathematical self-indulgence and are seldom read by ecologists, apart from those of us unfortunate enough to be asked to review them. However, *Modelling Fluctuating Populations* is exceptional in more than one way, and provided me with an unexpected pleasure; I enjoyed almost all of the book and found every chapter valuable.

Although there have been many books on mathematical ecology since Lotka's seminal contribution *Elements of Physical Biology* (Williams & Wilkins, 1925), few are memorable. May's *Stability and Complexity in Model Ecosystems* (Princeton University Press, 1973) is an exception and must be regarded as one of the most important books in the field; as well as some new insights into the subject matter of the title, the book provided a lucid account of the mathematical techniques used by physicists to analyse equations of motion. The main shortcoming of May's book was the deliberate omission of stochastic models, apart from the use of Kolmogorov's diffusion equation to represent a particular type of environmental variability.

In *Modelling Fluctuating Populations*, Nisbet and Gurney have given a thorough and rigorous account of the techniques available for investigating the dynamic properties of models, both deterministic and stochastic. Just as important, they devote some space to the problem of formulating the most appropriate mathematical representation of a process. Most ecologists will find the mathematics hard to follow, but not impossibly so; and at least the authors do not pretend that difficulties both in manipulation and interpretation do not exist.

The presentation of text and equations is of a very high quality, but unfortunately the same cannot be said of the lettering of the figures, many of which call for the use of hand-lens — in this respect the book is more suitable for the field ecologist than the theoretician. Some of the worst examples are where material has been taken from other publications and photoreduced (e.g. Figs 2.15 and 2.16), but there are many other such instances among the figures drawn especially for the book (e.g. Figs 2.9, 5.8 and 7.13). It is a pity that an otherwise excellent production is marred

by such an elementary but important flaw.

Like many before them, Nisbet and Gurney find themselves in deep water when they try to put their ecological models into an evolutionary context. Stability is a property of populations and is not a direct consequence of natural selection, and Nisbet and Gurney are honest enough to invoke group selection explicitly when they suggest why limit cycles might be rare in nature. Leaving aside the difficult question of what conditions are necessary for group

selection to be of any importance, the authors have a clear penchant for point equilibria rather than stable limit cycles, and most of their linear approximations require the existence of a stable equilibrium point. However, they do not shirk the task of coping with non-linearity where necessary as evidenced by their interesting treatment of Nicholson's blow-fly in Chapter 8. This chapter, and the other two case-studies, are very worthwhile and in themselves provide adequate justification for the rest of this excellent book. □

Robert H. Smith is a Lecturer in Applied Zoology at the University of Reading.

The evolution of landscapes

J. Rose

Climatic Geomorphology. By Julius Büdel. Pp.528. Hbk ISBN 0-691-08294-4; pbk ISBN 0-691-08295-2. (Princeton University Press: 1982.) Hbk £40.62, \$65; pbk £15.31, \$24.50.

OVER the past few decades geomorphology in most English-speaking countries has primarily dealt with the interaction between particular surface processes and the resisting surface materials. For the sake of practicality most of these studies have been concerned with isolated problems, river-channel bedforms or hillslope hydrology for example. The results of this work have been summarized in systematic texts such as Sugden and John's *Glaciers and Landscape* (Edward Arnold, 1976) and Schumm's *Fluvial System* (Wiley, 1977).

Similarly, where English-speaking geomorphologists have investigated the evolution of landscapes through time they have largely adopted an interdisciplinary approach within the framework of the Quaternary; again, these studies have been the subject of significant texts such as Goudie's *Environmental Change* (Oxford University Press, 1977) and Bowen's *Quaternary Geology* (Pergamon, 1978). An explanation for the wider span of landform assemblages and integrated landform development has received little attention other than in Pitty's *Introduction to Geomorphology* (Methuen, 1971), and this has been far from widely understood. *Climatic Geomorphology* by Julius Büdel, now Emeritus Professor of Geography at Würzburg, Germany is an unashamed attempt to remedy this deficiency.

Büdel's view of geomorphology consists of an investigation of the processes involved, which he calls "active geomorphology"; an appreciation of the role and magnitude of contemporary landscape alteration, which he terms "dynamic geomorphology"; and the global stage upon which the processes take place, to which he gives the title "climato-genetic geomor-

phology". "Climatic geomorphology" is the foundation for all these approaches and is the thesis of this book.

In many respects *Climatic Geomorphology* is a personal expression of over 50 years' geomorphological experience from all regions of the globe. Although there are some distinctive personal traits, such as a degree of contempt for the isolated studies that have dominated geomorphological research in the English-speaking world, it is far from out of date, with some 38 per cent of the references published in the decade before it went to press, and far from parochial with 19 per cent of the references in the English language. The book is divided into three parts: an introductory section which explains the geomorphic processes, interactions and spheres of operation in a concise but illuminating fashion; a main body of text which reviews the global landscape in terms of morphogenetic regions; and a series of case-studies from selected areas to illustrate the principles so developed. This last section gives particular emphasis to aspects of the central European landscape.

To a considerable extent, Büdel's approach to geomorphology, and hence the rationale behind his book, can be understood in terms of his desire to explain the landscape of central Europe with geomorphologically active Alpine mountains and geomorphologically quiescent incised valleys and upland plains. The mountain and glacial morphogenetic zones he considers to be globally ubiquitous and therefore receive little attention. The incised valleys are the goal of his study of "ectropical" regions and the results of fieldwork in Spitzbergen lead him to believe that bedrock disruption and disaggregation by ground ice beneath river channels provide the mechanism for "excessive valley deepening". Although the argument is clear and well illustrated it is, in my opinion, far from convincing and much of the evidence put forward by Büdel for con-

temporary *erosion* in his research area is not valid, but is the product of contemporary *deposition* in a region of aggrading ground ice. His quest for an explanation for geologically discordant plateaux takes him to the "peritropical" zones of contemporary etchplain development which he sees as the process for the formation of extensive, low-relief plains. To most of the British geomorphologists interested in cold-climate processes his descriptions of weathering and wash processes in humid tropical regions give a most illuminating explanation for our much-abused and little-understood erosion surfaces.

In many ways, Büdel's views must make geomorphologists in the English-speaking world reassess the results of their single-case, analytical studies. For instance, it is

interesting to consider if the approach taken by the contributors to Derbyshire's *Geomorphology and Climate* — published in 1976 (by Wiley) one year before *Climatic Geomorphology* appeared in Germany — gives a greater insight into how landforms and landscapes develop, if it achieves less or if it says the same thing in different ways. For what it is worth, my views are that the recent efforts of English-speaking geomorphologists have contributed much that is of significance to their subject, and that much of Büdel's criticism is unjustified. Equally, however, I believe that without the wisdom shown by Büdel's synthesis many of the individual analytical studies will be wasted.

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expect few readers to be disappointed.

The manual emphasizes both the biological systems and biochemical tools available. Each topic is introduced clearly, though concisely, taking care to equip the reader with some understanding of the systems and techniques he may use. Quite elementary information is included and a motivated, and perhaps green-fingered, novice should be able to start from simple experiments and rise to the more sophisticated technology, given, of course, the availability of a battery of good enzymes. Even here advice is offered about the careful use and preservation of enzymes. Although the volume is a laboratory manual it is not lacking in basic theoretical information, is well supplied with references and is amply provided with clear illustrations.

The authors have covered the predicted range of topics including vector-host systems, gel electrophoresis, isolation of mRNA, cDNA cloning, the construction of gene libraries and the detection and analysis of recombinant molecules. The information is supplemented with useful appendices full of recipes and simple biochemical methods. In some areas the manual is up to the minute, particularly in its presentation of a recombination method for screening bacteriophage lambda libraries for specific DNA sequences. In contrast it denies the usefulness of bacteriophage λ as an efficient means of cloning cDNA despite contrary documentation in the literature early in 1981.

One criticism that I would make of this otherwise excellent manual, a criticism that possibly reflects the pressures of a fast-moving field, is the presence of more errors than one usually associates with Cold Spring Harbor publications. Thus a list of around 20 *E. coli* strains includes at least five mistakes. Some of these — for example, *supF* where it should be *supE* — are likely to confuse the beginner who will assume that he himself, and not the manual, is in error. In similar vein, *λsep6-lac5* is stated to be exceptional in having the *red* and *gam* genes in its right arm; in fact, it is not exceptional and furthermore, since it is *gam*⁺ and *red*⁺ (not *red*⁺) it may be propagated on a *recA*⁺ host in the absence of both phage and host recombination systems.

Despite such blemishes I strongly recommend this manual. Those working in the area of molecular cloning, or contemplating doing so, may have received their copy already; others should put their name on the waiting list. □

Noreen Murray is Reader in the Department of Molecular Biology at the University of Edinburgh.

Nature publishes an annual review of new journals. This year's New Journals Review appeared in the issue of 7 October.

Bonding chemistry unravelled

B.F.G. Johnson

Multiple Bonds Between Metal Atoms. By F. Albert Cotton and Richard A. Walton. Pp.466. ISBN 0-471-04686-8. (Wiley: 1982.) \$63.20, £39.50.

I ADMIRE few chemists as much as F.A. Cotton. He has had a long and successful career and became one of the few inorganic chemists to achieve international prominence, both through the excellence of his research and because he can always be counted on to provide a clear description and cogent explanation of his chemistry. He is a highly skilled author and together with Dick Walton has written a classic book on multiple bonds between metal atoms. This (in my opinion) is Cotton's favourite topic and it shows.

The book examines the fundamental chemistry of compounds containing metal-metal bonds with bond orders greater than one. The study of such compounds has contributed much to modern inorganic chemistry. Unravelling the bonding problems provided by their structures has provided a wonderful stimulus to researchers, from which a totally new and exciting branch of chemistry has emerged.

"Introduction and Survey", the first of the eight chapters in the book, provides a fascinating insight into the genesis of this area of research and how it continues to progress. Apart from this and a further chapter devoted to physical, spectroscopic and theoretical results, the book deals with the formation of multiple bonds found between metal atoms (rhenium, molybdenum, tungsten and rhodium), and with their synthesis, structure and chemistry. Each chapter has been carefully sub-divided into small packages of information, thereby providing a concise and easily followed account. By so doing those of us concerned with compiling a succinct lecture course or simply locating

information relevant to our own research will have few problems. There is a wealth of good diagrams and tables, the literature survey is first class and the overall standard of production is excellent.

Cotton and Walton have provided a clear insight into the chemistry and importance of multiple metal-metal bonds. They have written a valuable and entertaining mix of chemistry, reminiscence and opinion, and have thereby given us an enjoyable, readable book which is a must for all practising inorganic chemists. □

B.F.G. Johnson is Reader in Inorganic Chemistry at the University of Cambridge.

Everyman's guide to molecular cloning

Noreen E. Murray

Molecular Cloning: A Laboratory Manual. By T. Maniatis, E.F. Fritsch and J. Sambrook. Pp.545. Pbk ISBN 0-87969-136-0. (Cold Spring Harbor Laboratory: 1982.) \$40 (US), \$48 (elsewhere).

FOR ten years the Cold Spring Harbor publication *Experiments in Molecular Genetics* by J.H. Miller has been a popular and reliable reference book for those working with *Escherichia coli* and its phages. I had anticipated that the laboratory manual *Molecular Cloning* would serve a similar role for those using *E. coli* as host and its plasmids and phages as vectors for the cloning and analysis of DNA. The long awaited manual is now available and I

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JUSSAWALLA, M. and LAMBERTON, D.M. (eds). Communication Economics and Development. Pp.345. ISBN 0-08-027520-6. (Pergamon: 1982.) £15, \$30.

KRUCKEBERG, A.R. Gardening with Native Plants. Pp.252. ISBN 0-295-95893-6. (University of Washington Press: 1982.) £17.50.

LEWTAS, J. (ed.). Toxicological Effects of Emissions from Diesel Engines, Vol.10. Proceedings of the Environmental Protection Agency Symposium held October 1981, North Carolina. Pp.380. ISBN 0-444-00687-7. (Elsevier/North Holland Biomedical: 1982.) \$65, Dfl.170 (outside N. America).

MALIK, M.A.S., TIWARI, G.N. *et al.* Solar Distillation. Pp.130. ISBN 0-08-028679-8. (Pergamon: 1982.) £12.50, \$25.

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ROFFMAN, R.A. Marijuana as Medicine. Pp.156. Hbk ISBN 0-914842-71-4; pbk ISBN 0-914842-72-2. (Madrona, Seattle: 1982.) Hbk \$11.95; pbk \$5.95.

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SOLOMON, E. Beyond the Turning Point: The US Economy in the 1980s. Pp.157. Hbk ISBN 0-7167-1390-X; pbk ISBN 0-7167-1391-8. (Freeman: 1982.) Hbk £10.40; pbk £5.50.

SRIVASTAVA, H.M. and KASHYAP, B.R.K. Special Functions in Queuing Theory, and Related Stochastic Processes. Pp.308. ISBN 0-12-660650-1. (Academic: 1982.) \$37.50.

THOMAS, E. The Heart of England. Pp.228. Pbk ISBN 0-19-281353-6. (Oxford University Press: 1982.) £2.95.

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WYANT, W.K. Westward in Eden. Pp.536. ISBN 0-520-04377-4. (University of California Press: 1982.) £17.50, \$31.85.

ANNOUNCEMENTS

Appointments

Dr Vincenzo Buonassisi has been appointed deputy director of the W. Alton Jones Cell Science Centre.

President Reagan has nominated **Mr Robert Gilkeson**, **Dr William Miller** and **Dr William Nierenberg** to serve for six-year terms as members of the National Science Board, the policymaking body of the National Science Foundation.

The British Secretary of State for Foreign and Commonwealth Affairs has appointed the **Hon John D. Eccles** as a member of the board of the Commonwealth Development Corporation for three years.

Professor J. Lloyd and **Mr G. Telling** have been appointed as members of the Food Additives and Contaminants Committee. **Dr W. Elstow**, and **Professor P. Turner** have been re-appointed as members of the Committee.

Mr Nigel Lawson, the British Secretary of State for Energy, has appointed six new directors for Britoil: **Mr Michael Kelly**, **Mr Larry Tindale**, **Sir Archie Lamb**, **Mr Ralph Quartano**, **Sir Alistair Frame**, and **Sir Kenneth Corfield**.

Dr Paul Winson has been made marketing manager for the Oxford Instruments Group, UK.

Techne (Cambridge) Limited have recently appointed a new sales and marketing manager, **Mr M.B.C. Sharp**. Mr Sharp has taken over the complete marketing function embracing both UK and export sales.

Mr Peter James has been appointed managing director of Fisons scientific equipment division's Australian subsidiary Townson & Mercer.

Awards

The Hammer Prize Foundation has awarded its first \$100,000 prize for cancer research to **Ronald Levy** of Stanford University, USA and **George Stevenson** of the University of Southampton, UK. They were cited for their work on monoclonal antibodies in the treatment of B-cell leukaemia and lymphoma.

Three industrialists have been elected trustees of Scripps Clinic and Research Foundation, the biomedical research and health care institution in La Jolla, California. These are **David Clare** president of Johnson & Johnson, **Richard Cramer** chairman and chief executive officer of IMED Corporation and **Gordon Anderson** formerly with Baker International, now in private investments.

Professor Vernon Mountcastle, University Professor of Neuroscience at the Johns Hopkins University School of Medicine, has been awarded the Hermann von Helmholtz Award for distinguished research in the cognitive neurosciences. It is the first to be made by the Cognitive Neuroscience Institute of New York.

The Prize Jury of the Novo Foundation of Denmark has decided to award the Novo Prize 1983 to **Professor Christian Crone** of the Panum Institute, University of Copenhagen. The Novo Prize is given as a reward for a major achievement, medical or otherwise, which may benefit the medical science. Professor Crone receives the Prize in recognition of his great contributions towards the understanding of capillary functions.

The winners of the first EPIC award — Education in Partnership with Industry and Commerce — have been announced. Joint first prize winners are the **University of Oxford** in conjunction with **Oxford Instruments Group of Industries** and the **Heriot-Watt University** with **Edinburgh Instrument Limited**. The Heriot-Watt/Edinburgh Instruments partnership won their prize for the development of gas laser and opto-electronic devices. The Oxford winners will use their prize of £25,000 to further develop scanning techniques that do not use X-rays and other applications of superconducting magnets. Runners up for the EPIC award, all of whom received £2,000, were: **Cambridge University** in conjunction with **TAL Chemicals Company** for their development of a fruit coating to control the rate at which fruit ripens; **Liverpool University** and **Rentokil** for the improved manufacture of Rentokil Supatimber preservatives and the development of a new process for the manufacture of arsenic acid; **UMIST** and **Froude Engineering Ltd** for the production of new dynamometers, devices used in the development of automotive engines; and the **University of Wales** plus **Lion Laboratories** for developments in their 15 year long collaboration over the measurement of alcohol in breath samples.

The only fellowship awarded this year by the British Mental Health Foundation has been won by **Dr Susan Wonnacott**, a biochemist at Bath University. The fellowship, worth £40,000 over a 3-year period, will enable Dr Wonnacott to continue her work on nicotinic receptors in the brain, with a view to investigating Alzheimer's disease, a progressive senile dementia which affects large numbers of elderly people. This condition is characterized by a progressive deterioration of mental function, primarily in thought and memory and secondarily in feeling and conduct.

The Bristol-Myers Award for distinguished achievement in nutritional research has been given to **Dr Elsie May Widdowson** of Addenbrooke's Hospital, Cambridge, UK. It is an annual award of \$25,000, and this year is the first time that the award will be given to a researcher from the UK.

The Mullard Award has been presented this year to **Oxford Instruments** for their work on advanced superconducting magnets and associated systems, particularly in the area of Nuclear Magnetic Resonance.

A project to develop credit transfer and improve advice on courses available to students has been launched by the Department of Education and Science. A contract, worth nearly £900,000 over three years, has been awarded to the **Open University** to develop and implement the piloting of a computerised Educational Counselling and Credit Transfer Information Service (ECCTIS) in the South West of England.

The Foulkes Foundation, a registered charity which aims to further medical research, has announced the winners of the Foulkes Foundation Fellowship. The fellowship gives financial support to recently qualified science graduates with research experience who want to study medicine, and medical graduates who want to take a science degree. The successful candidates for 1982 are: **Dr D.S. Blanchard**, **Dr J.P. Burrows**, **Dr D.A. Gray**, **Dr P.M. Matthews**, **Dr R. Silcock**, **Mr K.A. Steer**, **Dr C.-K.V. Tse** and **Dr E. Walker**. Entries for the 1983 awards are invited. Application forms will be available from the Fellowship Registrar in January 1983 and applicants are asked to enclose a stamped, addressed envelope.

Entries are invited for the Prince of Wales Award for Industrial Innovation and Production. The Award was first inspired by His Royal Highness in 1980 and aims to identify promising new products or processes and encourage the inventor or entrepreneur to get them into production and onto the market as soon as possible. It is being organized by The Engineering Council. The closing date for receipt of entries is 28 February 1983. Entry forms and further details may be obtained from: The Engineering Council, Canberra House, 10-16 Maltravers Street, London WC2R 3ER, UK.

ICI has made an endowment of £10,000 to a trust fund which will be administered by The Royal Society of Chemistry, to organise a biennial symposium on industrial chemistry in memory of Dr Alfred Spinks, CBE.

The Ciba Foundation is establishing a bursary scheme linked to the Ciba Foundation symposia. This scheme will enable young scientists to attend Ciba Foundation symposia and subsequently spend a period in the laboratory of one of the major participants. The bursars will be selected from laboratories or institutes not represented at the symposium. The successful bursar's travel, accommodation and other expenses will be met by the award.

Two National Institute on Ageing teaching nursing home grants, totalling \$7.43 million over five years, have been made available to support research on geriatric health problems in a nursing home setting. The National Institute on Ageing is part of the US Public Health Service. These are the first grants of their kind to be proposed.

Nominations are invited for the Marconi International Fellowship. Nominees should be individuals whose work in the fields of communication science and technology exemplifies the technical creativity and concern for human welfare of Guglielmo Marconi. The deadline for nominations is 15 April 1983. Final selection of the recipient will be made in October 1983 and the successful candidate will receive £35,000. The Fellowship Selection Committee will give special attention to the identification of emerging fields of communications science and technology. Nominators are asked to specify why they believe that the nominee has made innovative contributions to a field of research or technology of growing future significance.

British Gas has set up a Teaching Fellowship at Cambridge University attached jointly to the Engineering and Chemical Engineering Departments. **Mr. N.K. Macfadyen** has been appointed as its first holder.

Meetings

10-12 January, **The Brain, Biochemistry and Behaviour**, Florida (Ms M. Hack, 355 St Paul's Avenue, Staten Island, NY 10304, USA).

16-22 January, **International Seminar on Human Waste Management**, Bangkok, (Ms T. Liamzon, Seminar Co-ordinator, PO Box 24-130, Bangkok 10240, Thailand).

20 January, **Innovation Conference**, Manchester (Mr J. Jackson, Shirley Institute, Manchester M20 8RX, UK).

January, **Conference on the Palaeoenvironment of East Asia**, Hong Kong (R.O. Whyte, University of Hong Kong, Centre of Asian Studies, Pokfulam Road, Hong Kong).

6-10 February, **Third Annual Congress for Recombinant DNA Research**, Philadelphia (Mr E. Ruffing, Scherago Associates, 1515 Broadway, Tenth Floor, New York, NY 10036, USA).

6-10 February, **Second Annual Congress for Hybridoma Research**, Philadelphia (Mr E. Ruffing, Scherago Associates, 1515 Broadway, Tenth Floor, New York, NY 10036, USA).

6-10 February, **"Biotech '83"**, Philadelphia (Mr E. Ruffing, Scherago Associates, 1515 Broadway, Tenth Floor, New York, NY 10036, USA).

7-11 February, **Optical Methods in Engineering Metrology**, Colchester (The Liaison Officer, Laser Workshop Courses, University of Essex, Colchester CO4 3SQ, Essex, UK).

7-11 February, **Course in Gear Design**, Sheffield (Dr H.K. Kohler, Director for Engineering and Design Courses, Department of Mechanical Engineering, University of Sheffield, Mappin Street, Sheffield S1 3JD, UK).

13-15 March, **First International Risk Seminar**, London (Ms D.E. Jarvis, PO Box 62, Beaconsfield, Bucks HP9 2NY, UK).

4-5 April, **Wind/Solar Energy Conference**, Kansas City (G.H. Stickney, College of Engineering, University of Missouri-Columbia, Columbia, MO 65211, USA).

5-8 April, **Electroanalysis in Biomedical, Environmental and Industrial Sciences**, Cardiff (Dr J.D.R. Thomas, Applied Chemistry Department, Redwood Building, UWIST, Cardiff CF1 3NU, UK).

9-11 April, **Interfacial Kinetics in Solution**, Hull (Professor D.H. Everett, Department of Physical Chemistry, School of Chemistry, Cantock's Close, Bristol BS8 1TS, UK).

11 April, **Opioids: Past, Present and Future**, Cambridge (Dr M.B. Tyers, Neuropharmacology Department, Glaxo Group Research Limited, Greenford Road, Greenford, Middlesex UB6 0HE, UK).

11-14 April, **International Conference on Combustion in Engineering**, Oxford (Institute of Mechanical Engineers, 1 Birdcage Walk, Westminster, London SW1H 9JJ, UK).

11-14 April, **7th UK Geographical Assembly**, Leicester (M.A. Khan, Department of Geology, University of Leicester, University Road, Leicester LE1 7RH, UK).

3 May, **International Symposium on Crop Protection**, Gent (The Secretary, Faculteit van de Landbouwwetenschappen, Coupure Links 533, B-9000 Gent, Belgium).

3-6 May, **Canadian Symposium on Remote Sensing**, Montreal (R. Desjardins, Department de Geographie, UQAM, BP 8888, Succ A, Montreal H3C 3P8, Canada).

5 May, **Energy Saving in the Chemical Industry**, London (Rubber and Plastics Research Association, Shawbury, Shropshire SY4 4NR, UK).

8-13 May, **Prevention of Occupational Accidents and Diseases**, Ottawa-Hull (The Xth World Congress on the Prevention of Occupational Accidents and Diseases, c/o Canadian Organising Committee, 500-300 Slater Street, Ottawa, Ontario, Canada K1P 6A6).

9-12 May, **International Conference and the Third Annual Meeting of the Electrophoresis Society**, Tokyo (Secretariat Electrophoresis '83, c/o Dr Nobuya Hashimoto, Department of Internal Medicine, The Jikei University School of Medicine, 3-25-8, Nishishimbashi, Minato-ku, Tokyo 105, Japan).

10-12 May, **Progress in Perinatal Medicine**, Florence (P.G. Crosignani, IVth Department of Obstetrics and Gynaecology of the University of Milan, Via M. Melloni, 52-Milan, Italy).

10-21 May, **Course on Measurement, Analysis and Control of Noise**, Sheffield (Dr H.K. Kohler, Director for Engineering and Design Courses, Department of Mechanical Engineering, University of Sheffield, Mappin Street, Sheffield S1 3JD, UK).

16-20 May, **International Conference on Radioactive Waste Management**, Seattle (International Atomic Energy Agency, Wagramerstrasse 5, PO Box 100, A-1400 Vienna, Austria).

17-18 May, **Heat and Fluid Flow in Nuclear and Process Plant Safety**, London (The Institution of Mechanical Engineers, 1 Birdcage Walk, Westminster, London SW1H 9JJ, UK).

17-20 May, **Automated Manufacturing Exhibition and Conference**, Birmingham (Clapp and Poliak Europe Ltd, 232 Acton Lane, London W4 5DL, UK).

17-20 May, **Conference and Exhibition of Lasers and Electro-Optics (CLEO '83)**, Baltimore (Publicity Chairman, CLEO '83, Tiemo von Zweck, Laser Power Optics, 11211 Sorrento Valley Road, San Diego, California, USA).

23-27 May, **International Conference on Graphite Intercalation Compounds**, Pont-a-Mousson, France (Dr Daniel Guerard, Laboratoire de Chimie Minerale Appliquee, Université de Nancy I, BP 239, 54 506 Vandoeuvre les Nancy, France).

24-26 May **International Conference on Road Vehicle Handling**, Nuneaton (The Institution of Mechanical Engineers, 1 Birdcage Walk, Westminster, London SW1H 9JJ, UK).

24-26 May, **International Conference on Ecology and Environmental Quality**, Jerusalem (Professor H. Shuval, Chairman, Israel Ecological Society, Hebrew University-Hadassah Medical School, PO Box 1172, Jerusalem, Israel).

24-26 May, **Energy Storage**, Stratford-Upon-Avon (Conference Organiser, Energy Storage, BHRA Fluid Engineering, Cranfield, Bedford MK43 0AJ, UK).

25 May, **The Ciba Foundation 34th Annual Lecture**, London (Miss J. Holman, The Ciba Foundation, 41 Portland Place, London W1N 4BN, UK).

31 May-1 June, **Course on Water Supply and Sanitation for Developing Countries**, Ottawa (Dr R. Droste, Water Sanitation Short Course, Department of Civil Engineering, University of Ottawa, Ottawa, Ontario, Canada K1N 9B4).